# Elucidating the metabolic pathways responsible for higher alcohol production in *Saccharomyces cerevisiae*

by

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# Declaration

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# Summary

Alcoholic fermentation, and especially wine fermentation, is one of the most ancient microbiological processes utilized by man. Yeast of the species *Saccharomyces cerevisiae* are usually responsible for most of the fermentative activity, and many data sets clearly demonstrate the important impact of this species on the quality and character of the final product. However, many aspects of the genetic and metabolic processes that take place during alcoholic fermentation remain poorly understood, including the metabolic processes that impact on aroma and flavour of the fermentation product. To contribute to our understanding of these processes, this study took two approaches:

In a first part, the initial aim had been to compare two techniques of transcriptome analysis, DNA oligo-microarrays and Serial Analysis of Gene Expression (SAGE), for their suitability to assess wine fermentation gene expression changes, and in particular to assess their potential to, in combination, provide combined quantitative and qualitative data for mRNA levels. The SAGE methodology however failed to produce conclusive data, and only the results of the microarray data are shown in this dissertation. These results provide a comprehensive overview of the transcriptomic changes during model wine fermentation, and serve as a reference database for the following experiments and for future studies using different fermentation conditions or genetically modified yeast.

In a second part of the study, a screen to identify genes that impact on the formation of various important volatile aroma compounds including esters, fatty acids and higher alcohols is presented. Indeed, while the metabolic network that leads to the formation of these compounds is reasonably well mapped, surprisingly little is known about specific enzymes involved in specific reactions, the genetic regulation of the network and the physiological roles of individual pathways within the network. Various factors that directly or indirectly affect and regulate the network have been proposed in the past, but little conclusive evidence has been provided. To gain a better understanding of the regulations and physiological role of this network, we took a functional genomics approach by screening a subset of the EUROSCARF strain deletion library, and in particular genes encoding decarboxylases, dehydrogenases and reductases. Thus, ten

genes whose deletion impacted most significantly on the aroma production network and higher alcohol formation were selected. Over-expression and single and multiple deletions of the selected genes were used to genetically assess their contribution to aroma production and to the Ehrlich pathway. The results demonstrate the sensitivity of the pathway to cellular redox homeostasis, strongly suggest direct roles for Thi3p, Aad6p and Hom2p, and highlight the important role of Bat2p in controlling the flux through the pathway.

# Opsomming

Alkoholiese fermentasie, en veral die maak van wyn, is een van die vroegste mikrobiologiese prosesse wat deur die mensdom ingespan is. Die gisspesie *Saccharomyces cerevisiae* is gewoonlik grotendeels verantwoordelik vir die fermentasie and verskeie vorige studies het gedemonstreer dat hierdie spesie ‘n baie belangrike rol speel in die uiteindelike kwaliteit en karakter van die voltooide produk. Nieteenstaande die feit is daar steeds baie aspekte van beide die genetiese en metaboliese prosesse wat plaasvind tydens alkoholiese fermentatsie wat nog swak verstaan word, insluitende metaboliese padweë wat ‘n impak het op die smaak en aroma van die fermentasie produk. Om ons kennis van die veld uit te brei het die studie twee aanslae geneem:

In die eerste geval is gepoog om twee tegnieke van transkriptoom analiese, nl. DNA oligo-mikro-arrays en Serial Analysis of Gene Expression (SAGE) te bestudeer vir hul vermoë om geen ekspressie veranderinge tydens wynfermentasie te ondersoek en meer spesifiek om hul potensiaal om ‘n kombinasie van kwantitatiewe sowel as kwalitatiewe data met betreking to mRNA vlakke te produseer. Die SAGE metode kon egter geen betroubare resultate produseer nie en dus word slegs die resultate van die mikro-array eksperimente in die tesis bespreek. Die resultaat is ‘n geheeloorsig oor die geenekspressie veranderinge wat so ‘n wyngis tydens alkoholiese fermentasie ondergaan en dien as ‘n verwysingsraamwerk vir toekomstige studies met geneties gemodifiseerde gis of selfs verskillende fermentasieparameters.

Die tweede deel van die studie het gefokus op die identifikasie van gene wat ‘n impak het op die vorming van belangrike, vlugtige aroma komponente, o. a. Esters vetsure en hoër alkohole d.m.v. ‘n siftingseksperiment. Alhoewel daar redelik baie inligting is oor die onderligende metaboliese netwerke wat lei tot die vorming van die verbindings, is daar min kennis van die genetiese regulasie van die netwerk en die fisiologiese rol van individuele padweë wat die netwerk vorm. Verskeie faktore – wat of die netwerk direk of indirek affekteer – is al voorgestel, meer met min konkrete bewyse. Dus het ons gepoog om meer lig op die onderwerp te laat m.b.v. ‘n funksionele genoom aanslag deur ‘n siftingseksperiment te doen op ‘n subgroep (spesifiek gene wat kodeer vir dekarboksilase, dehidrogenase en reduktase ensieme) van die

EUROSCARF delesiebiblioteek. Dus is tien gene geïdentifiseer – die delesie waarvan ‘n merkbare effek het op die aroma produksie netwerk en spesifiek die van hoër alkohole. Ooruitdrukkings en enkel en meervoudige delesie rasse van die tien gene is gemaak om d.mv. genetiese analiese, hulle rol in aroma produksie en die Ehrlich padweh uit te pluis. Die resultate toon dat hierdie padweg sensitief is teenoor die sellulêre redoks balans en dui op direkte rolle vir Thi3p, Aad6p en Hom2p, asook dat Bat2p ‘n baie belangrike rol speel in die werking van die padweg.

This dissertation is dedicated to my parents

# Biographical sketch

Gustav Styger was born in Roodepoort on the 3rd of October 1975. He attended Laerskool Gustav Preller and matriculated in 1993 from Florida Hoërskool. Gustav obtained a B.Sc. *cum laude* degree (Biochemistry, Zoology and Botany) from the Rand Afrikaans University (now known as the University of Johannesburg) in 1996. In 1997 he obtained the degree B.Sc. Honours *cum laude* (Biochemistry) from Stellenbosch University and in 2001 the degree M.Sc. *cum laude* (Biochemistry) from the same institution.

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# Preface

This dissertation is presented as a compilation of six chapters and an appendix. Each chapter is introduced separately and is written according to a general style as Chapter 4 will be submitted to the journal *Applied Microbiology and Biotechnology* and Chapter 5 will be submitted for publication to the journal *Applied and Environmental Microbiology*

**Chapter 1** **General Introduction and Project Aims**

**Chapter 2** **Literature Review**
Wine Flavour and Aroma

**Chapter 3** **Research results**
Transcriptome analysis of an industrial yeast strain during a model wine fermentation: Results from oligo-cDNA microarrays

**Chapter 4** **Research results**
Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols

**Chapter 5** **Research results**
Genetic analysis of the metabolic pathways responsible for higher alcohol production in *Saccharomyces cerevisiae*

**Chapter 6** **General discussion and conclusions**

**Appendix** Lilly M., Bauer F.F., Styger G., Lambrechts M.G. & Pretorius I.S., (2006) The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. *FEMS Yeast Research*. 6(5) 726-743

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# Chapter 1

## General Introduction and Project Aims

# 1. GENERAL INTRODUCTION AND PROJECT AIMS

## 1.1 INTRODUCTION

The perception of wine flavour and aroma is the result of a multitude of interactions between a large number of chemical compounds and sensory receptors. Compounds interact and combine and show synergistic (i.e. the presence of one compound enhances the perception of another) and antagonistic (a compound suppresses the perception of another) interactions. The chemical profile of a wine is derived from the grape, the fermentation microflora (and in particular the yeast *Saccharomyces cerevisiae*), secondary microbial fermentations that may occur, and the aging and storage conditions [1]. The chemistry of wine has been intensively studied and compounds have been identified that are responsible for certain individual varietal or readily identifiable aromas. These include the varietal characteristics of muscat wines [2, 3], the characteristic fruity or green bouquet of Sauvignon blanc wines [4, 5], as well as the peppery notes of Shiraz [6, 7]. However, it is mostly a combination of factors that impart the aroma of a wine.

Yeast can contribute to wine aroma by several mechanisms: firstly by utilising grape juice constituents and biotransforming them into aroma or flavour impacting components, secondly by producing enzymes that transform neutral grape compounds into flavour active compounds and lastly by the *de novo* synthesis of many flavour active primary- (e.g. ethanol, glycerol, acetic acid and acetaldehyde) and secondary metabolites (e.g. esters, higher alcohols, fatty acids acids) [8]. The aim of the study presented in this dissertation was to contribute to our understanding of *de novo* biosynthesis of secondary metabolites.

The biochemical pathways resulting in secondary metabolites form a heavily interconnected network and changes in one component or part of the pathway can impact on many other, a priori unrelated compounds [9]. It is thus clear that this metabolic network is complex and, while reasonably well mapped, little information about the role of specific genes and their regulation

within this network is available [10]. One specific and central pathway within this network is the Ehrlich reaction, the catabolic pathway whereby, amino acids, amongst others, the branched-chain amino acids valine, leucine and isoleucine, are metabolised into aroma compounds such as higher alcohols or volatile fatty acids [11]. Whether a higher alcohol or fatty acid is formed is thought to depend on the redox status of the cell, as the reaction towards a higher alcohol takes place via a NADH-dependent reaction. Thus isoamyl alcohol is formed from leucine, isobutanol is formed from valine and active amyl alcohol is formed from isoleucine [12-14]. The formation of a volatile fatty acid requires $NAD^+$ and so iso-valeric acid is formed from leucine, iso-butyric acid is formed from valine and 2-methyl butanoic acid is formed from isoleucine [12-14]. Little conclusive information about the specific enzymes responsible for these conversions has been published. It has in particular been suggested that an alcohol dehydrogenase may catalyze the reductive reaction and an aldehyde dehydrogenase the oxidation reaction [15, 16]. Other data suggest that the decarboxylation reaction taking place in the Ehrlich reaction could be catalysed by the pyruvate decarboxylase genes - *PDC1*, *PDC5* and *PDC6*, but they clearly appear not to be essential [17, 18]. Other possible decarboxylase genes that could be involved are *KID1/THI3* and *ARO10* [15, 16, 19]. However, no systematic analysis of this metabolic network has this far been undertaken.

## 1.2 PROJECT AIMS AND APPROACHES

This study is a continuation of work that had been undertaken by our group at the Institute for Wine Biotechnology regarding the catabolism of the branched-chain amino acids, leucine, isoleucine and valine and the impact of their metabolites, especially the higher alcohols, to the aroma profile wine fermentations [9]. In a previous study it was shown that the gene products of *BAT1* and *BAT2* were responsible for the first step in the pathway of branched-chain amino acid catabolism, also known as the Ehrlich reaction [11]. Changes in the concentrations of other, seemingly unrelated, aroma compounds were also observed, highlighting the complexities of the interconnections within such complex metabolic networks [9]. The main objectives of this

study was therefore to search for other genes that could impact on the aroma profile of yeast, with specific interest in those genes whose deletion had an impact on the formation of higher alcohols. An important part of the present study was also to perform a further in-depth study of these genes and to try and deduce how they impact on the formation of higher alcohols and related compounds.

In order to achieve this, the following approaches were taken. Firstly a small 10 litre model wine fermentation using a clearly defined synthetic wine medium (MS300) was established and used to reproduce the fermentation kinetics and characteristics of a wine fermentation using an industrial wine yeast strain, EC1118. Cell and medium samples were taken at regular intervals (every six hours during the first 24 hours, thereafter at twelve hour intervals) and to extract mRNA from the cells and analyse the medium for various compounds. These cellular samples were used for cDNA microarray analysis, comparing each time point in the fermentation to the preceding one in order to examine the transcriptomic regulation that the yeast cells undergo during such a model wine fermentation.

For the second part of the study, a large scale screen for genes – using deletion strains from the EUROSCARF deletion library, grown in synthetic complete dextrose medium (SCD) - that impact on the yeast aroma profile, with specific focus on genes that effected metabolites associated with the catabolism of the branched-chain amino acids, leucine, isoleucine and valine was undertaken. The ten deletion strains that showed the most significant impact on these products were identified using the following criteria: deletion of candidate genes had to lead to large decreases in the production of higher alcohols such as isoamyl alcohol and isobutanol, and possibly be associated with decreases in either iso-valeric acid or iso-butyric acid. After initial selection, these results were re-confirmed in SCD medium supplemented with branched-chain amino acids. Finally, independent deletion strains as well as over-expression strains were constructed and used to analyse the effect of these genetic perturbations on the metabolites associated with branched-chain amino acid catabolism, as well as the general

aroma profile of the strain when grown in branched-chain amino acid supplemented SCD medium.

The specific aims of this study were therefore:

1. To add to our understanding of the genetic network responsible for regulating the pathways responsible for aroma and flavour compound formation in *S. cerevisiae* with specific regards to the apparent interconnectedness of the yeast aroma production pathway.
2. To identify the specific genetic factors involved in these aroma production pathways with specific focus on the Ehrlich pathway and branched-chain amino acid catabolism in general by using a semi-directed gene deletion screening approach.
3. To investigate through genetic analysis the role of identified genes within the Ehrlich pathway, in particular by determining whether the observed effect was indirect, or the result of a direct role within the pathway.

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# Chapter 2

## Literature Review

### Wine Flavour and Aroma

# 2. WINE FLAVOUR AND AROMA

## 2.1 INTRODUCTION

The final sensorial quality of a wine is the result of a multitude of interactions between all the chemical components within the wine and specific environmental factors such as the temperature of the wine. The chemical composition of the wine is primarily dependent on the type and quality of the grapes. Viticultural practices for example aim primarily at producing quality grapes that would reflect varietal flavours and aromas and / or characters typical for a specific region or terroir. This involves harvesting grapes at specific stages of ripeness depending on the style of wine to be made. Once harvested, specific processing techniques and fermentation strategies that are implemented will further determine the aroma and flavour development of the wine. Beside some prefermentative treatments such as maceration, the composition of the microflora present in the grape must and in particular the wine yeast strain and the selective application of malolactic fermentation (MLF) are of significant relevance [1-4]. The final aroma and flavour profile is furthermore strongly dependent on all aspects of post-fermentation treatments such as filtration and maturation strategies, including for example the aging in wooden containers.

Once the product has been finalised, the appreciation of wine requires all five senses: firstly to observe the colour and appearance, secondly to judge the wine bouquet, thirdly to taste the wine itself and fourthly to enjoy the mouth-feel and aftertaste [5]. One might also include sound as a prerequisite to the complete enjoyment of wine – the uncorking of a bottle and the sound of pouring is unique to wine! However, for the purposes of this review only chemical compounds and associated sensorial properties with regards to wine flavour and aroma will be discussed. The major chemical components responsible for some of these sensory attributes are listed in **Table 2.1**.

The complexity of this system is further revealed by studies indicating that not only does the unique interaction between numerous chemical compounds determine the flavour and aroma of

**Table 2.1.** Sensory components and some of the major chemical compounds responsible for their attributes in wine and grapes. Table adapted from Polaskova, *et al*. [5]

| Sensory component | Attribute | Responsible chemicals in wine |
|---|---|---|
| Taste | Sweet | Glucose, fructose |
| | | Glycerol, ethanol |
| | Sour | Tartaric acid |
| | Salty | Sodium chloride |
| | | Potassium chloride |
| | Bitter | Catechin |
| Smell/Aroma | Floral, lily-of-the-valley aroma | Linalool |
| | Bannana-like aroma | Isoamyl acetate |
| Chemestesis | Mouth warming/Heat | Ethanol |
| Tactile | Viscosity | Glycerol, polysaccharides |
| | Fullness | Mannoproteins |
| | Astringency | Tannins |
| Vision | Red | Malvidin-3-glucoside |

a wine – with ethanol playing a very important role in this regard [6, 7] - but that certain physical and environmental aspects such as the temperature of the wine or even the shape of the wine glass can also greatly change the perception of aroma and flavour [8]. Although wine tasting and perception is therefore largely a subjective experience, and simple factors such as the absence or presence of saliva can greatly influence the release of aroma compounds from both red and white wines [9].

From a scientific perspective, sensory perception needs to be analysed by isolating specific impact factors. In a first analysis of a mixture of odorants it needs to be assessed which odorants are most important. This will depend on the threshold of an odorant and its concentration. A compound may have a high concentration but if its threshold is large (i.e. a high concentration of this compound is needed to smell it) it will not contribute significantly to the aroma. On the other hand, a compound with a low threshold and large concentration will probably dominate the aroma [10]. However, it has been shown that low impact odorants may act to change the perception of other odorants in a mixture, and may interact synergistically or antagonistically [11]. The odour activity value (OAV), or as it is sometimes called the flavour activity, is equal to the concentration of a component of the aroma divided by its detection threshold level [7].

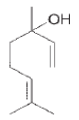
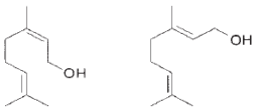
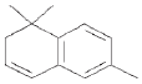
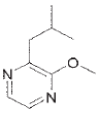
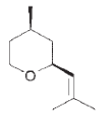
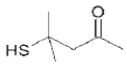
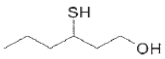
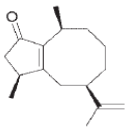
This review will try and give an overview of wine aroma and flavour starting with a section on the aromas associated with varietal character of grapes and wines. The main focus of the review deals with describing how metabolites associated with primary yeast metabolism, as well as those formed from secondary metabolic pathways can influence and establish wine aroma. More detail will be given on aroma compounds associated with amino acid catabolism, as this forms the basis of the rest of the study. The effect of malolactic fermentation and storage on wine flavour and aroma will also be discussed. A section on human perception of flavour and aroma will conclude the review.

## 2.2 VARIETAL FLAVOURS AND AROMAS

Although the overall composition of most grape varieties is much the same, there are clear and distinct aroma and flavour differences between some of these cultivars. However, these differences can mostly be attributed to slight variations in the relative ratios of the compounds that constitute the aroma profile of a wine, and there are only a few aroma compounds that have been directly linked to specific varietal flavours and aromas [5]. Some of these compounds and their characteristics are listed in **Table 2.2**. Although most of these compounds are present at low concentrations in both grapes and the fermented wine, they normally have a large odour activity value (OAV) and thus can have a huge impact on the overall aroma profile [12].

Varietal wine aroma from muscat-related grapes for example is mainly due to the presence of various isoprenoid monoterpenes in the grapes, with the most important being; linalool, geraniol, nerol, citronellol and homotrienol [13]. These compounds are formed from the precursor mevalonate, as shown in **Figure 2.1**, itself a metabolite derived from acetyl-CoA. The monoterpenes can be found in free and odourless glycosidically-bound form in grape berries and the ratio of free and bound forms changes during berry ripening, with mature berries showing more bound forms than free forms of these compounds [14, 15].

**Table 2.2.** Impact odorants contributing to varietal aromas of selected wines. Table adapted from Polaskova, *et al*. [5].

| Cultivar | Impact compounds | Odour characteristic | Sensory threshold | Structure |
|---|---|---|---|---|
| Muscat | Linalool | Floral | 170 ng/L (in water) |  |
| | Terpenols - geraniol<br>- nerol | Citrus, floral<br>Citrus, floral | |  |
| Riesling | TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) | Kerosene | 20 µg/L | |
| Sauvignon blanc | 3-Isobutyl-2-methoxypyrazines (IBMP) | Bell pepper | 20 µg/L (in water) |  |
| Gewurztraminer | *cis* -Rose oxide | Geranium oil | 200 ng/L |  |
| | Wine lactone | Coconut, woody, sweet | 0.02 pg/L (in air) |  |
| Sauvignon blanc | 4-Methyl-4-mercaptopentan-2-one | Blackcurrent | 0.6 ng/L |  |
| Sauvignon blanc,<br>Semillon | 3-Mercapto-1-hexanol | R isomer - Grapefruit, citrus peel<br>S isomer - Passion fruit | 50 ng/L<br>60 ng/L |  |
| Shiraz | Rotundone | Black pepper | 16 ng/L |  |

During fermentation yeast can release glucosidases and these enzymes can hydrolyse the glycosidic bonds of the odourless bound forms of monoterpenes, releasing more odour contributing compounds to the wine [16-18]. Studies have also shown that skin contact treatment can significantly increase the concentrations of both free and bound odour compounds [19]. It has been found, however, that the formation of varietal aroma is in fact an integral part of yeast metabolism and not a simple hydrolytic process, as previously thought [20]. Some studies even show that yeast can synthesize some of these monoterpenes in the absence of grape derived precursors [21]. Furthermore, the yeast strain and genus has been shown to have an important influence on the levels of most varietal aroma compounds, affecting

all families formed from precursor molecules, including C13-norisoprenoids, and monoterpenes [16].

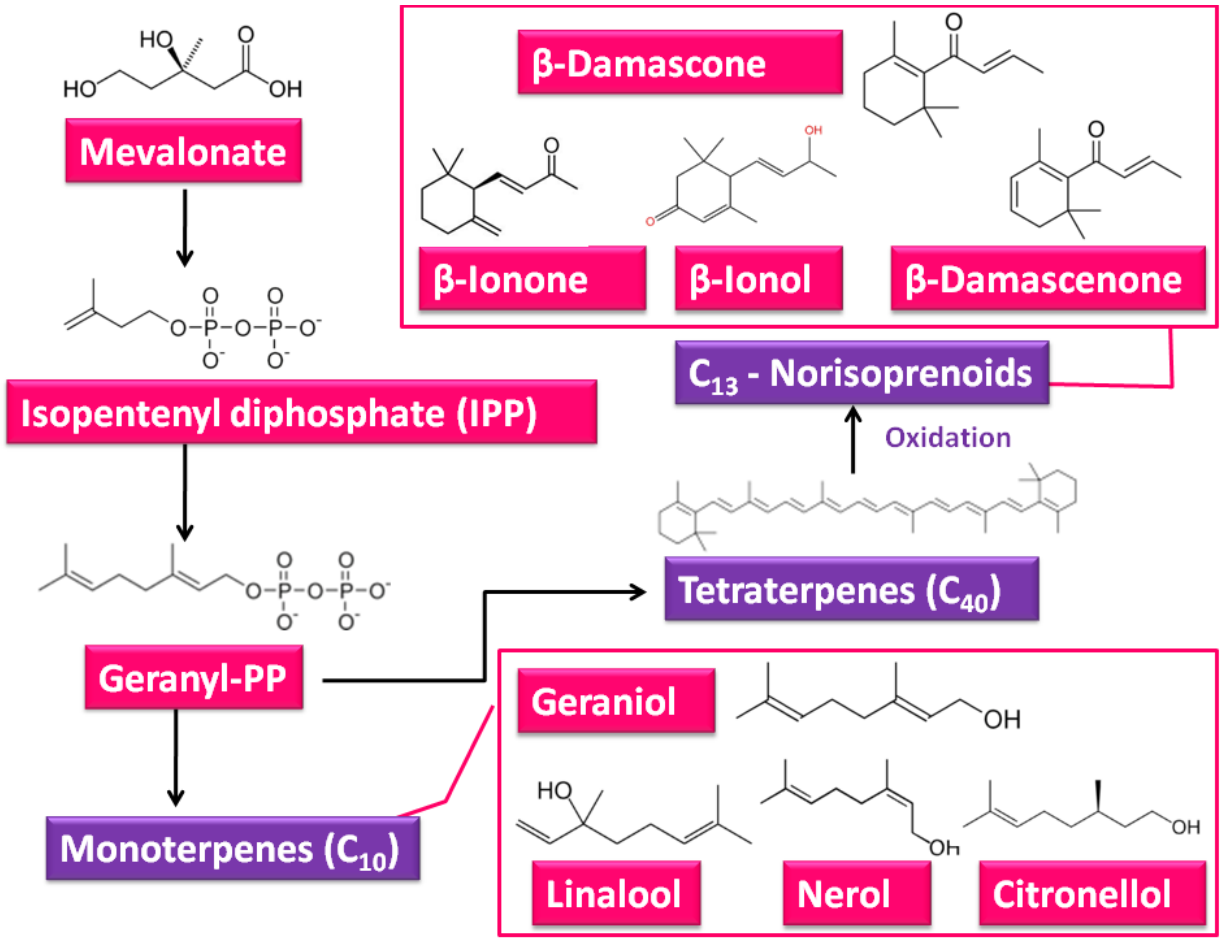



**Fig. 2.1.** Diagram indicating the formation of the most important chemical compounds responsible for varietal aroma in wine. Both monoterpenes and C13-norisoprenoids are formed from the precursor mevalonate, itself a metabolite of acetyl-CoA. Figure adapted from Iriti and Faoro [13].

Another set of varietal aroma compounds released from odourless bound precursors are those that give Sauvignon blanc wines their characteristic bouquet, i.e. 4-Methyl-4-mercaptopentan-2-one and 3-Mercapto-1-hexanol (see **Table 2.2**). These compounds are not present in grape juice in their active form, but odourless, nonvolatile, cysteine-bound conjugates occur in grape must and the wine yeast is responsible for the cleaving of the thiol from the precursor during alcoholic fermentation [22]. It is interesting to note that some varietal aromas occur completely independent of each other. It is thought that the ‘green’ characters in Sauvignon blanc wines

(imparted by the chemical compound 3-isobutyl-2-methoxypyrazines (IBMP) can be manipulated through vineyard management, however, the 'tropical fruity' characters appear to be largely dependent on the wine yeast strain used during fermentation and its ability to cleave the cysteinated-precursors with a carbon–sulphur lyase enzyme [23].

**Figure 2.1** also indicates the formation of a second set of compounds that play a role in varietal aroma i.e. carotenoids. These isoprenoids tetraterpenes also originate from the precursor compound mevalonate, where five carbon units are condensed. Oxidation of these carotenoids produces volatile and strong odour-contributing fragments known as C13-norisoprenoids, e.g. β-ionone (viola aroma), damascenone (exotic fruits), β-damascone (rose) and β-ionol (fruits and flowers) [13].

Another compound recently discovered that imparts a distinctive varietal pepper aroma to Shiraz wine (see **Table 2.2**) is the sesquiterpene, rotundone. Researchers identified the unknown peppery compound in white and black pepper and found that the same compound was responsible for the associated aroma and flavour in Shiraz wines. This disproved the previous hypothesis that this varietal pepper aroma was due to the complex interactions of many odorants, or to piperine and related alkaloids, which impart 'heat' in the mouth [24, 25]. However, the main source of aroma and flavour compounds found in the finished wine comes not from the grape, but rather from compounds formed during primary (essential) or secondary metabolism of the wine yeast during alcoholic fermentation. Some of the important compounds thus formed will be discussed below.

## 2.3 FLAVOURS AND AROMAS FORMED BY THE YEAST DURING FERMENTATION

According to Fleet (2003), yeast influence wine aroma by the following mechanisms: (i) The bio-control of moulds by yeasts before harvest – mainly by apiculate yeast species competing for nutrients, (ii) the alcoholic fermentation of the grape juice into wine, (iii) the de novo

biosynthesis of flavour and aroma compounds during alcoholic fermentation, (iv) the metabolism of flavour neutral grape compounds into active aroma and flavour compounds, (v) post fermentation impact on wine via autolysis, and (vi) influencing the growth of malolactic and spoilage bacteria [26]. Of these, the de novo biosynthesis of flavour and aroma compounds is probably the most important, since in general, fermentation-derived volatiles make up the largest percentage of total aroma composition of wine [5]. In **Figure 2.2** the formation of the most important of these aroma compounds is schematically represented. It is important to note that formation of these compounds are variable and yeast strain specific [27] and that this review will only deal with a generalised view of yeast aroma compound metabolism.

### 2.3.1 FLAVOURS AND AROMA COMPOUNDS DIRECTLY RELATED TO ALCOHOLIC FERMENTATION

As stated above, the aroma bouquet of a wine is a complex interaction between numerous volatile chemical compounds. These compounds interact with each other in various ways to achieve the final aroma and flavour palette. Quantitatively, metabolites that are direct products and by-products of glycolysis, the central carbon metabolism during alcoholic fermentation, are found in the highest concentration. These compounds include ethanol, glycerol and acetic acid. While usually presenting low OAVs, their high concentration makes them important impact compounds. Studies have shown that a reduction of the ethanol concentration in a model wine from 10% to 9% had no effect on the flavour or aroma profile. When the ethanol concentration was further lowered to 7%, a marked increase in the intensities of the fruity, flowery and acid flavours and aromas were seen. However, when the ethanol concentration was dropped to 3%, the model wine didn’t resemble wine anymore [7]. Another study showed that by reducing the alcohol levels in wine, one can affect the aromatic bouquet, not only by strengthening the perceived interactions between woody and fruity wine odorants, but also by modifying their chemical proportions [6].

Another very important flavour compound directly related to fermentation is glycerol. This compound was historically thought to be a major contributor to the overall mouth-feel of wine and higher glycerol concentrations were seen as advantages and can enhance the complexity

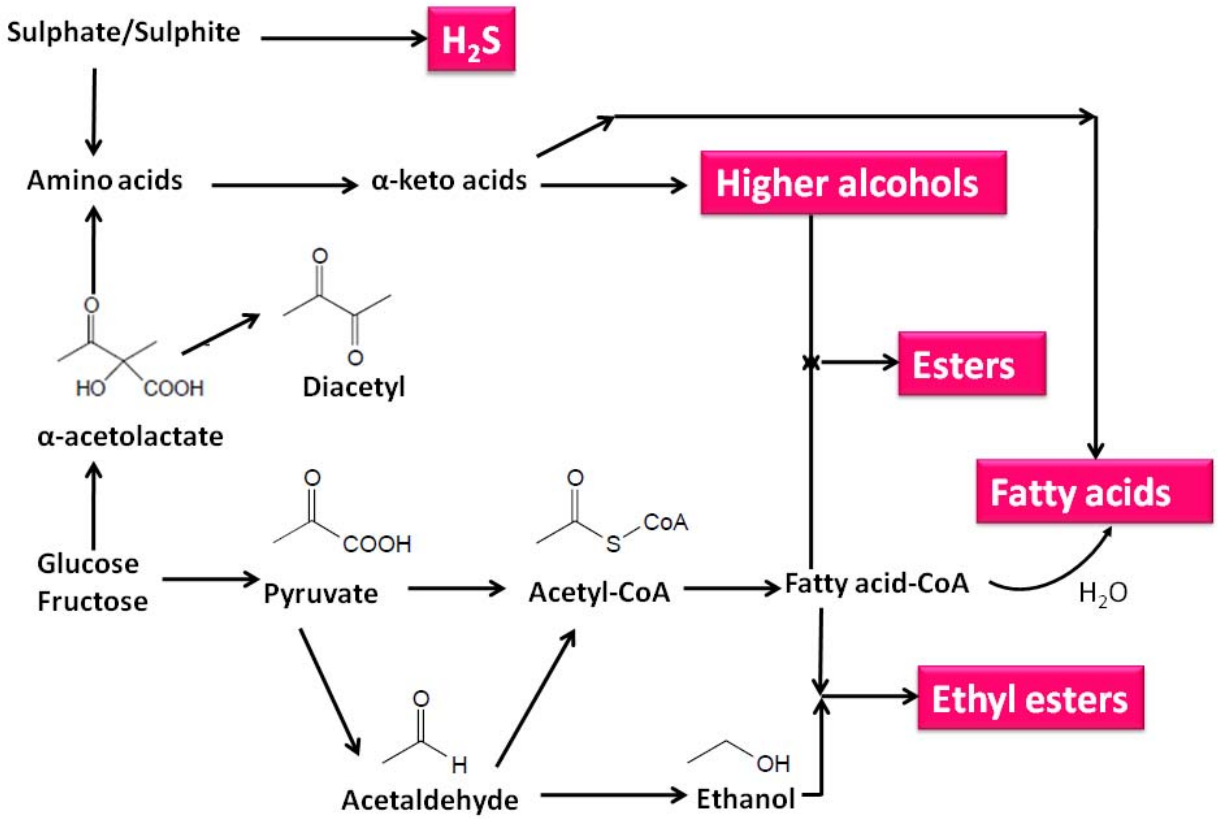



**Fig. 2.2.** A schematic representation of some of the major classes of aroma compounds produced by yeast during alcoholic fermentation. Figure adapted from Lambrechts and Pretorius [27]

of the wine. Normally dry wines contain about 4 – 10 g/l of glycerol [28]. However, little attention has been given to the interaction of glycerol and various flavour compounds and the role that glycerol plays in the formation of the aroma profile. Earlier studies with sensory analysis showed that the perceived overall flavour profile of a model wine and a white wine was not changed by the addition of glycerol and that glycerol does not play a part in establishing the aroma bouquet of wine [29]. However, recent data suggests that while no statistical association exists between

glycerol concentration and quality of red wine, there is a significant statistical association between the concentration of glycerol and the perceived quality of all styles of white wine [30].

Acetaldehyde is also an important aroma compound formed from pyruvate (**Figure 2.2**) during vinification and constitutes more than 90% of the total aldehyde content of wine [31]. Acetaldehyde is also a precursor metabolite for acetate, acetoin and ethanol. It has been found that acetaldehyde levels reaches a maximum when the rate of fermentation is at its fastest, then decreases towards the end of fermentation, only to slowly increase again thereafter [27]. At low levels this compound imparts a pleasant fruity aroma to wine and other beverages, but at higher concentrations this turns into a pungent irritating odour reminiscent of green grass or apples [32]. Acetaldehyde is also extremely reactive and readily binds to proteins or individual amino acids to generate a wide range of flavour and odour compounds [33].

An important odorant formed from acetaldehyde is diacetyl. The formation of this compound is illustrated in **Figure 2.3**. Diacetyl is mainly formed by lactic acid bacteria during malo-lactic fermentation, but yeast are also able to synthesise this compound during the alcoholic fermentation. However, the majority of this diacetyl is further metabolised to acetoin and 2,3 butanediol [34]. Diacetyl at low concentrations (threshold value, 8 mg/litre) adds yeasty, nutty, toasty aromas to wine, but at high concentrations, it has a characteristic buttery aroma that is associated with a lactic character [34, 35]. Once again this compound is highly reactive and has been found to react with cysteine, forming sulphur compounds that can influence wine aroma [36]. Neither acetoin and 2,3 butanediol have a strong odour, with their threshold values of about 150 mg/litre in wine [35].

However, many important aroma compounds are not directly related to the central carbon metabolism pathway. These so-called secondary metabolites can be obtained from the metabolism of amino acids or fatty acids and the formation, and their effect on wine aroma, of the most important compounds are described below.

### 2.3.2 FLAVOURS AND AROMA COMPOUNDS RELATED TO AMINO ACID METABOLISM

During alcoholic fermentation yeast can use amino acids in several ways. In particular, they can be used as such for protein synthesis, or metabolized into other compounds and used for other purposes and metabolic processes [37]. However, worldwide studies have shown that most grape musts contain insufficient amounts of yeast nutrients, especially assimilable nitrogen. Such deficiencies are seen as some of the main causes for sluggish and stuck fermentations and nitrogen supplementation of grape musts has become common practice [38].

The nitrogen composition of the grape must affects not only the kinetics of alcoholic fermentation, but also the production of aromatic compounds, ethanol and glycerol [39]. It has even been shown that the varietal aroma character of certain cultivars could be partially explained by the amino acid profile in the grape must [40]. The two main sources of yeast assimilable nitrogen are primary amino acids and ammonium [39]. Although yeast strains differ greatly in their ability to use nitrogen and amino acids [41], various studies have shown that nitrogen supplementation in the form of assimilable nitrogen and amino acids have influences on the volatile aroma profile of the wine [38, 42-44].

Amino acids are transported into the cell by general and specific transport systems. The general high-capacity amino acid permeases like Gap1p [45-49] and Agp1p [47] are used for general transport, whilst the proline permease Put4p, the histidine permease Hip1p, the lysine permease Lyp1p and the basic-amino-acid permease Can1p are used for specific amino acids or classes of amino acids [47]. The branched-chain amino acids, leucine, isoleucine and valine are transported into the yeast cell via the high affinity branched-chain amino acid permease Bap2p [50-55], the closely related branched-chain amino acid permease Bap3p [56-58] and the tyrosine transporter Tat1p [57].

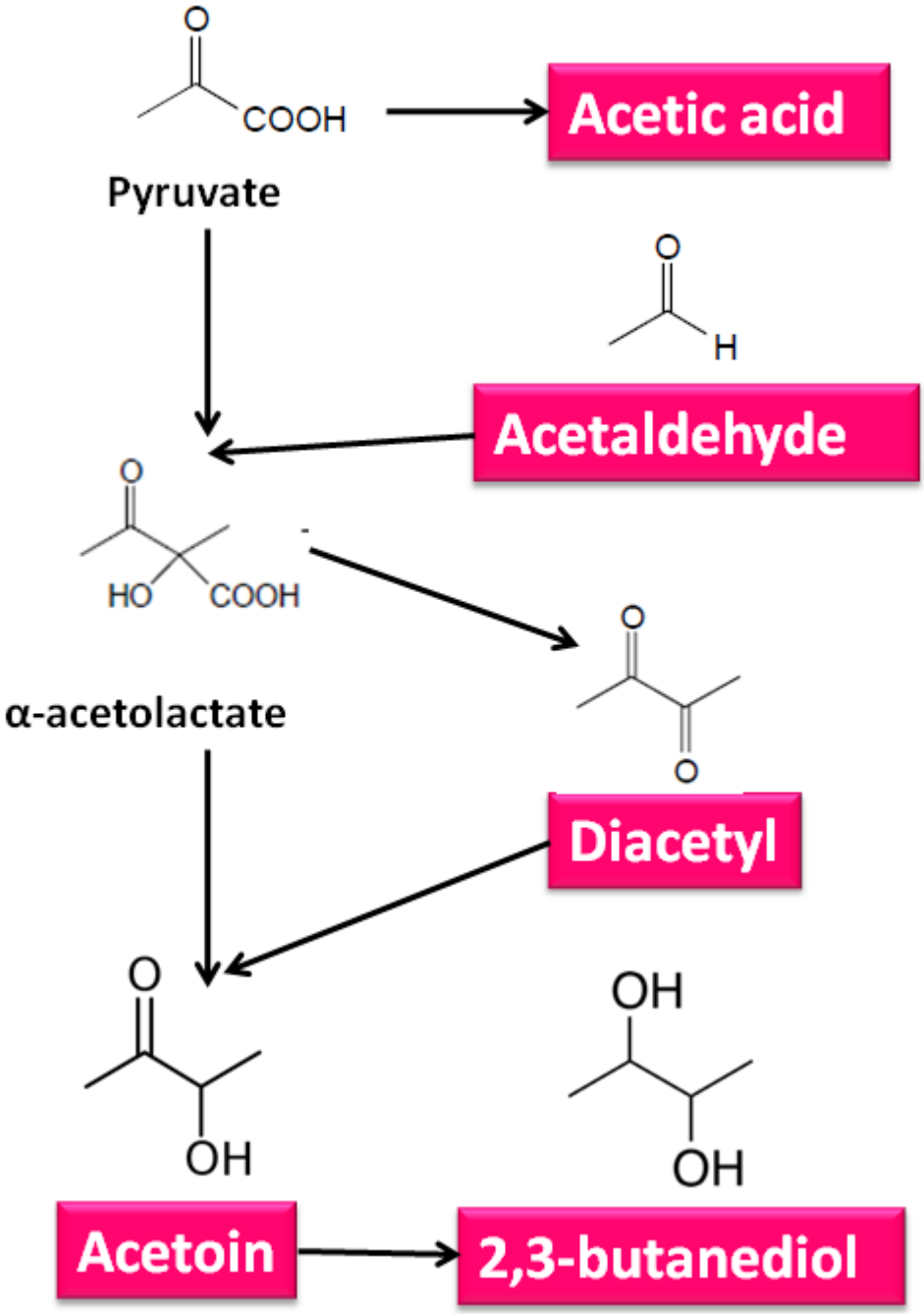



**Fig. 2.3.** A schematic representation the formation of diacetyl, acetoin and 2,3-butanediol by yeast during alcoholic fermentation. Figure adapted from Bartowsky and Henschke [34].

The most important flavour and aroma compounds formed from amino acids are higher alcohols and volatile acids. The process by which amino acids are catabolised into higher alcohols is called the Ehrlich reaction, indicated by the blue coloured reactions and compounds in **Figure 2.4** [59]. The Ehrlich reaction also impacts on various other aroma compounds, whether directly

or indirectly, as shown in **Figure 2.4** [60, 61]. In addition to the three branched-chain amino acids (**Table 2.4**), other amino acids can also be broken down to other metabolites via this reaction [62]. However, as the most important odour related products are produced from the branched-chain amino acids (see **Table 2.3**) the discussion will mainly focus on them. The first step in the pathway is a transamination reaction where the amino group from the amino acid is transferred to α-ketoglutarate to form an α-keto acid and glutamate [63-65]. In this manner α-ketoisocaproic acid is formed from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine, as well as phenyl puruvate from phenylalanine, *p*-hydroxy-phenyl puruvate from tyrosine, indole pyruvate from tryptophane, α-keto-butyrate from methionine and oxaloacetate from aspartic acid [62, 66-69]. The transamination reaction for the branched-chain amino acids is catalysed by mitochondrial and cytosolic branched-chain amino acid aminotransferases (BCAATases) encoded by the *BAT1* and *BAT2* genes [60, 70-74]. The Aro9p has been implicated in the transaminase reaction of the aromatic amino acids tryptophan, tyrosine and phenylalanine [75]. Yeast, however, can also generate these α-keto acids through the so-called anabolic pathway, from glucose via pyruvate [66, 67, 76].

**Table 2.3.** Branched-chain amino acid metabolites and their odour characteristics. Concentration and threshold values obtained from Lambrechts *et al*. [27].

| Compound | Amino acid | Concentration in wine (mg/l) | Threshold (mg/l) | Flavour |
|---|---|---|---|---|
| Isovaleraldehyde | Leucine | Traces | - | Green, malty |
| Isobutyraldehyde | Valine | Traces | - | Slightly apple-like |
| 2-Methylbutyraldehyde | Isoleucine | - | - | Green, malty |
| Iso-butyric acid | Valine | Traces | 8.1 | Sweet, apple-like, sweaty |
| Iso-valeric acid | Leucine | < 3 | 0.7 | Rancid, cheese, rotten fruit |
| 2-Methylbutanoic acid | Isoleucine | - | - | Fruity, waxy, sweaty fatty acid |
| Isoamyl alcohol | Leucine | 45 - 490 | 300 | Alcohol |
| Isobutanol | Valine | 40 - 140 | 500 | Fruity, alcohol, solvent-like |
| Active Amyl alcohol | Isoleucine | 15 - 150 | 65 | Marzipan |

**Table 2.4.** Flavour producing amino acid catabolism in anaerobic environment starting with aminotransferase activity. Table adapted from Ardö [62].

| Amino acid | α-keto acid | Aldehydes | Alcohols | Carboxylic acids | Others |
|---|---|---|---|---|---|
| Leucine | α-keto-iso-caproate | 3-Methylbutanal | 3-Methylbutanol | 3-Methylbutanoic acid | |
| Isoleucine | α-keto-β-methylvaleate | 2-Methylbutanal | 2-Methylbutanol | 2-Methylbutanoic acid | |
| Valine | α-keto-isovalerate | 2-Methylpropanal | 2-Methylpropanol | 2-Methylpropanoic acid | |
| Phenylalanine | Phenyl puruvate | Phenylacetaldehyde | Phenylethanol | Phenylacetic acid | |
| Tyrosine | *p*-OH-phenyl puruvate | *p*-OH-phenylacetaldehyde | *p*-OH-phenyl-ethanol | *p*-OH-phenylacetic acid | *p*-Cresol |
| Tryptophan | Indole pyruvate | Indole-3-acetaldehyde | Tryptophol | Indol-3-acetic acid | Skatole |
| Methionine | α-keto-butyrate | 3-Methylthiopropanal | 3-Methylthio-propanol | 3-Methylthiopropionic acid | Methanethiol |
| Aspartic acid | Oxaloacetate | | | Malate | Diacetyl, acetion |

The second step in the Ehrlich pathway is the decarboxylation of the α-keto acid into an aldehyde [77]. Thus, isovaleraldehyde is formed from α-ketoisocaproic acid, isobutyraldehyde from α-ketoisovaleric acid and 2-methyl butyraldehyde is formed from α-keto-β-methylvaleric acid as well as phenylacetaldehyde from phenyl puruvate, *p*-hydroxy-phenylacetaldehyde from *p*-hydroxy-phenyl puruvate, indole-3-acetaldehyde from indole pyruvate and 3-methylthiopropanal from α-keto-butyrate [62, 66-68, 78]. It has been suggested that the pyruvate decarboxylase genes - *PDC1*, *PDC5* and *PDC6*, may play a part in this decarboxylation reaction, but they are not essential [79, 80]. Other genes that could possibly be involved in the decarboxylation of these α-keto acids are *KID1/THI3* and *ARO10* [81-83].

The Ehrlich pathway now splits in two and the final fate of the amino acid is thought to depend on the redox status of the yeast cell [82]. The aldehyde can either be reduced via a NADH-dependent reaction to its respective higher alcohol, i.e. isoamyl alcohol is formed from isovaleraldehyde, isobutanol is formed from isobutyraldehyde and active amyl alcohol is formed from 2-methyl butyraldehyde as well as phenylethanol from phenylacetaldehyde, *p*-hydroxy-phenyl-ethanol from *p*-hydroxy-phenylacetaldehyde, tryptophol from indole-3-acetaldehyde and 3-methylthio-propanol from 3-methylthiopropanal [62, 66-68]; or it can be oxidized via a $NAD^+$ dependent reaction into a volatile acid. If this occurs, iso-valeric acid is formed from isovaleraldehyde, iso-butyric acid is formed from isobutyraldehyde and 2-methyl butanoic acid is formed from 2-methyl butyraldehyde as well as phenylacetic acid from phenylacetaldehyde, *p*-hydroxy-phenylacetic acid from *p*-hydroxy-phenylacetaldehyde, indol-3-acetic acid from

tryptophol, 3-methylthiopropionic acid from 3-methylthiopropanal [62, 66-68]. It has been suggested that an alcohol dehydrogenase may catalyze this reductive reaction and an aldehyde dehydrogenase the oxidation reaction [81, 83].

However, the Ehrlich pathway is not the only way that amino acids can be metabolised into flavour and aroma compounds. In **Figure 2.5**, alternative routes for the catabolism of threonine, methionine and aspartic acid are shown. Aspartic acid (panel C) can be deaminated to form oxaloacetate and it has been shown that some bacterial strains can further catabolised it into acetoin, diacetyl and 2.3-butanediol (discussed above) [62]. It is however, not known whether any yeast strains can complete this reaction. Threonine (panel A) can also be converted into the important odorant acetaldehyde and further into ethanol or acetic acid [62]. Methionine can be catabolised to release methanethiol following a demethiolation reaction. Methanethiol can be further converted to other sulphur compounds, and it could also react with carboxy acids to

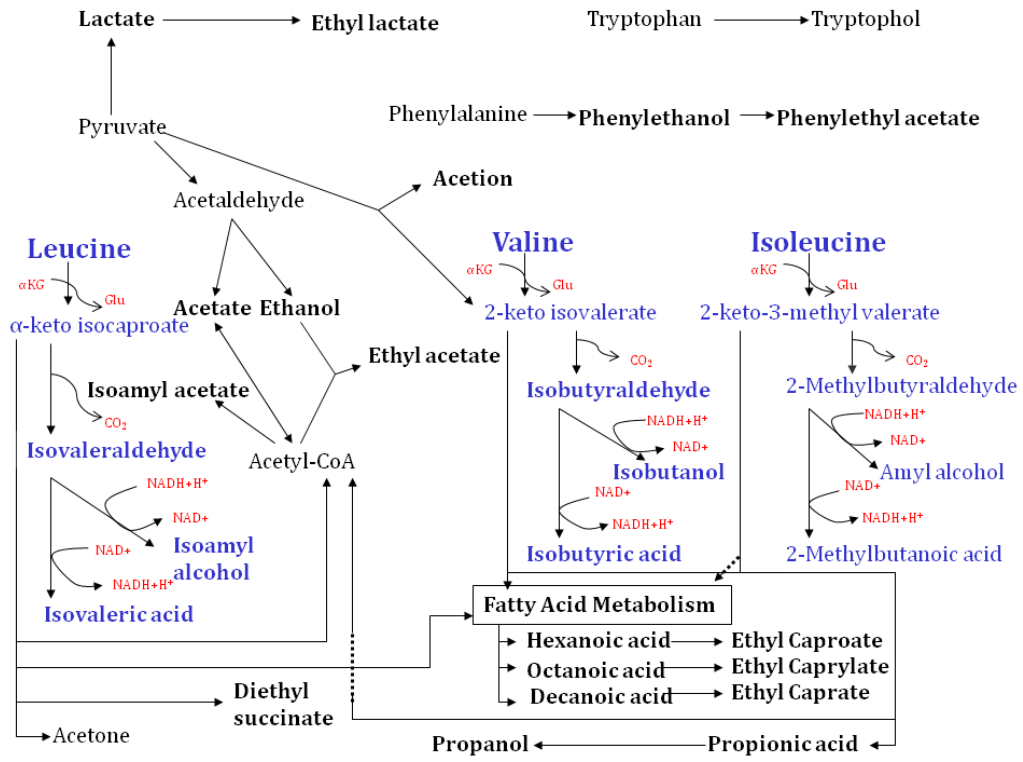



**Fig. 2.4.** A simplified metabolic map of yeast aroma compound production, indicating known metabolic linkages. Bold type indicates aroma compounds important to this study. Compounds shown in blue colour constitute a diagrammatical representation of the Ehrlich pathway, responsible for the production of higher alcohols and volatile acids. Co-factors and transition metabolites are shown in red. αKG is α-keto glutamate and Glu is glutamate [60, 61]

produce thioesters [84]. Another amino acid, cysteine can form various odour-impacting compounds through the so-called Maillard reaction, in which a chemical reaction between amino and carbonyl groups takes place to form new compounds [85].

### 2.3.3 OTHER FLAVOUR AND AROMA COMPOUNDS

Volatile esters constitute one of the most important classes of aroma compounds and is largely responsible for the fruity aromas associated with wine and other fermented beverages [86]. The enzyme-free formation of esters is an equilibrium reaction between an alcohol and an acid, however, it was found that this manner of ester formation is to slow to account for the large amounts of esters normally found in wine [27]. The enzymatic formation of esters was therefore identified as an initial activation of the acid by combining it with coenzyme A (CoA) before reacting with the alcohol to form an ester [27]. The coenzyme donor can either be acetyl-CoA (formed from pyruvate) or any of a range of acyl-CoA compounds formed by the enzyme acyl-CoA synthetase [87]. Thus the fatty acid, or ethyl esters (such as ethyl butanoate, ethyl hexanoate, ethyl octanoate) are formed from ethanolysis of acyl-CoA which is an intermediate metabolite of fatty acid metabolism. In this group of esters the ethanol group is derived from ethanol and the acid group from a medium chain fatty acid [88, 89]. The other group of esters, the acetate esters (such as isoamyl acetate, propyl acetate, hexyl acetate, phenethyl acetate) are the result of the reaction of acetyl-CoA with alcohols that are formed from the degradation of amino acids, carbohydrates, and lipids [88, 89].

This enzymatic synthesis has been widely studied in *Saccharomyces cerevisiae* during wine fermentation and various enzymes have been identified as playing a role in the formation of acetate esters, i.e. alcohol acetyltransferases – *ATF1* and *ATF2*, isoamyl alcohol acetyltransferase and ethanol acetyltransferase [86, 90, 91]. The formation of the ethyl esters has been attributed to the following two acyl-CoA:ethanol *O*-acyltransferase enzymes, Eeb1p and Eht1p [89]. While not important in the context of wine fermentation, other means of ester formation also exists. Examples of these reactions include oxidation of hemiacetal compounds

(formed from alcohol and aldehyde mixtures) by alcohol dehydrogenases and the reaction of a ketone with molecular oxygen by a Baeyer-Villiger monooxygenase [87].

However, the formation of esters differs widely from yeast strain to yeast strain and other external factors such as fermentation temperature, nutrient availability, pH, unsaturated fatty acid/sterol levels, and oxygen levels all play an important part in determining the end levels of esters in a wine [88, 92-94].

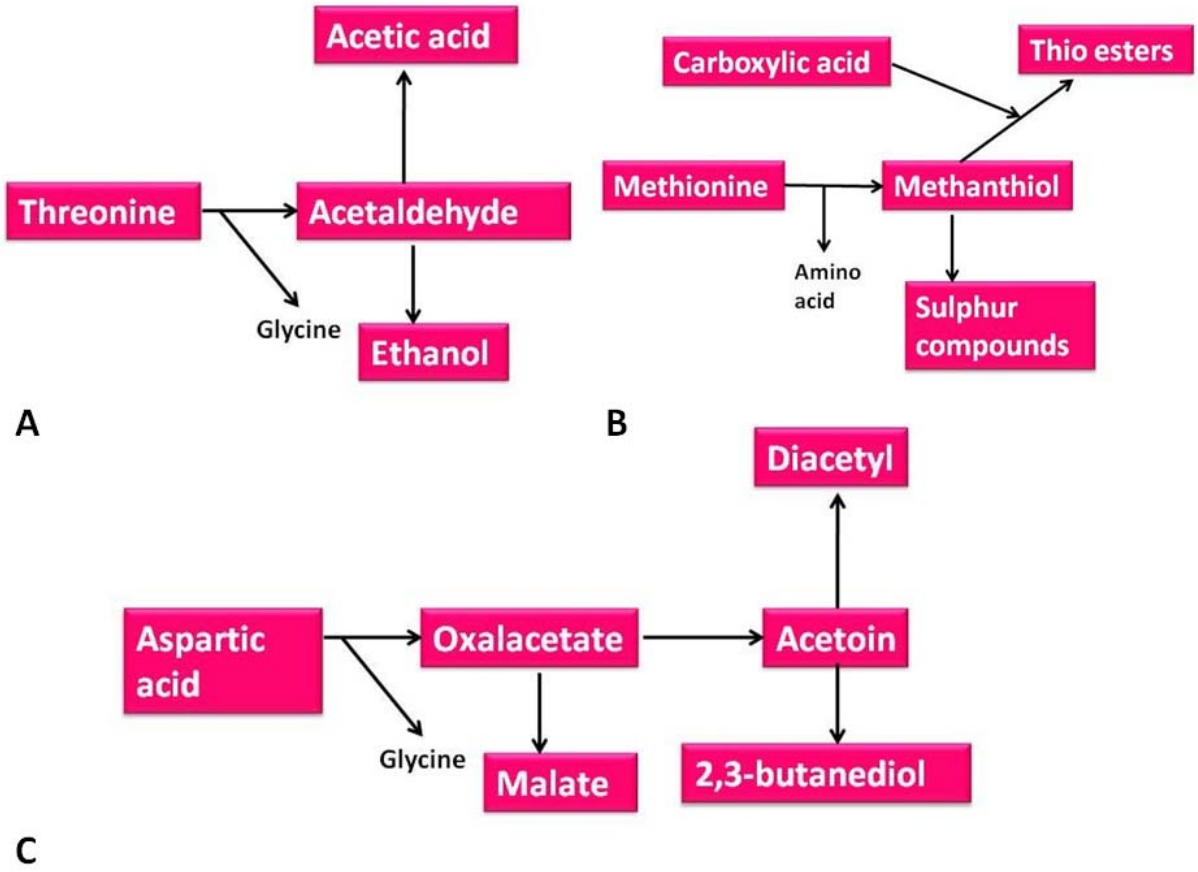



**Fig. 2.5.** Schematic representation of the catabolism of three amino acids into compounds important for wine flavour and aroma. Panel A represents threonine, panel B represents methionine and panel C represents aspartic acid. Figure adapted from Ardö [62]

Volatile fatty acids also contribute to the flavour and aroma of wine. The products of the branched-chain amino acids have already been discussed, but during the fermentation many medium and long chain-length fatty acids are also formed via the fatty acid synthesis pathway from acetyl-CoA [31]. Medium-length chain fatty acids are thought to be toxic to the yeast cells and retard fermentation, but studies on stuck and sluggish fermentations have shown that high levels of these medium chain-length fatty acids in these types of fermentations are symptomatic, rather than causative [95].

During wine fermentation *Saccharomyces cerevisiae* is not the only micro-organism that can contribute to the aroma and flavour of wine. Spontaneous fermentations will involve many non-*Saccharomyce*s species and some can impart novel aromas to the wine [96]. In the next section the effect of lactic acid bacteria and specifically malolactic fermentation on wine aroma will be discussed.

## 2.4 FLAVOURS AND AROMA COMPOUNDS FORMED DURING MALOLACTIC FERMENTATION

After alcoholic fermentation, some wines can undergo a secondary fermentation known as malolactic fermentation (MLF). This biological process is particularly desirable for high-acid wine produced in cool-climate regions, as MLF involves the deacidification of wine via the conversion of dicarboxylic L-malic acid (malate) to the monocarboxylic L-lactic acid (lactate) and carbon dioxide (**Figure 2.6**). This process is normally carried out by lactic acid bacteria (LAB) isolated from wine, including *Oenococcus oeni*, *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp [97]. MLF is also important in some wines from warmer regions since it changes the composition of the wine and improves its organoleptic quality. Moreover, it has been found that bacterial activity plays a role in the stabilisation of wine and ensures an enrichment of its aromatic composition [98].

During MLF LAB can influence the aroma and flavour of wine by producing volatile metabolites and modifying aroma compounds derived from grapes and yeasts. Similarly to the role that yeast plays in aroma formation, the effects of these bacteria are strain specific and can vary greatly [99]. Generally it has been found that MLF can enhance the fruity aroma and buttery note but reduce the vegetative, green/grassy aroma of wine. Additionally, flavour characteristics ascribed to wines undergoing MLF include: floral, nutty, yeasty, oaky, sweaty, spicy, roasted, toasty, vanilla, smoky, earthy, bitter, ropy and honey. Besides aroma, MLF is also believed to increase the body and mouthfeel of wine and give a longer after-taste [97].

Many LAB posses catalytic enzymes capable of liberating grape-derived aroma compounds from their natural non-aromatic glycosylated state [100]. Some of these enzymes include β-glucosidases, proteases, esterases, citrate lyases and phenolic acid decarboxylases. All of these classes of enzymes can possibly hydrolyse flavour precursors and so influence wine aroma [101-103]. It is interesting to note that many malolactic fermentations take place in oak barrels. Recent studies suggest that the LAB can also influence wine flavour and aroma by producing additional oak-derived compounds [104]. It was observed that the concentration of vanillin, a powerful aroma compound, increased during MLF in oak barrels. This finding suggests the existence of a vanillin precursor in the wood that is modified by LAB to release vanillin into the wine [105, 106].

LAB can also produce or decrease aroma impacting compounds via their own metabolism. It is thought that the enhanced fruitiness of wines that underwent MLF is due to the formation of esters by the LAB [97]. Much study is still needed on the effect of ester production by LAB during MLF, but evidence indicate that ethyl esters, such as diethyl succinate, ethyl acetate, ethyl lactate, ethyl hexanoate and ethyl octanoate, are formed during MLF [104]. During storage of the wine, it has been observed that concentrations of certain esters increase, whilst those of others decrease. This is thought to be due to acid hydrolysis and chemical esterification [97]. The concentration of another chemical compound important in wine aroma, acetaldehyde (see section 2.3.1) can be affected by the metabolism of LAB. It was shown that some species,

especially *Oenococcus oeni* can catabolise this compound, resulting in the formation of ethanol and acetate, with a subsequent reduction in the vegetative, green/grassy aroma of some wines [97]. However, the most important impact that MLF has on wine aroma is the increased buttery note of wines. This is mainly the result of the production of

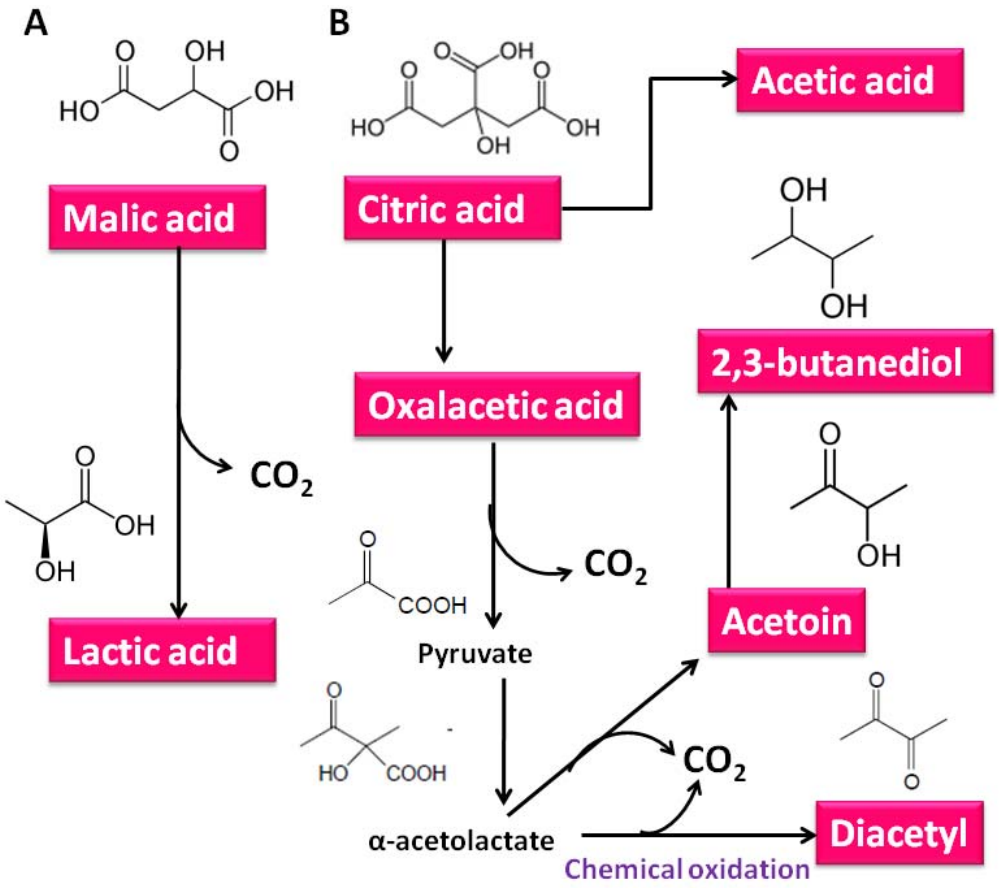



**Fig. 2.6.** Schematic representation of the two most important biochemical reactions catalised by lactic acid bacteria during malolactic fermentation. Panel A represents the conversion of dicarboxylic L-malic acid (malate) to the monocarboxylic L-lactic acid (lactate) and carbon dioxide, whilst the production of carbonyl or acetonic compounds, including diacetyl, acetoine, and 2,3 butanodiol from the metabolism of citric acid is represented in panel B.

carbonyl or acetonic compounds, including diacetyl, acetoine, and 2,3 butanodiol from the metabolism of citric acid by LAB via several reactions in which citrate lyase plays a role (**Figure 2.6**) [98]. Another recent study also showed that *O. oeni* can metabolise the amino acid methionine, resulting in the production of aroma and flavour impacting sulphur-containing compounds such as methanthiol, methyl disulphide, and methionol 3-(methylsulfanyl) propionic acid [98].

Brief mention was made above of the changes that wine can undergo during storage and maturation. The next section of the review will focus on these issues and their implications on wine aroma and flavour.

## 2.5 FLAVOURS AND AROMA COMPOUNDS FORMED DURING AGEING AND MATURATION

As described above, wine aroma and flavour is generated through an immensely complex interaction of various classes of aroma compounds and various environmental and biological factors. However, wine is also a dynamic product that undergoes a period of ageing or maturation, be it in the bottle or in oak barrels. This provides another level of complexity as reactions between the oak cask wall and the wine can change the chemistry of the wine [31]. Additionally, the structural characteristics of the wood, i.e. the grain, porosity and permeability, and its chemical composition, including polyphenols, tannins and volatile compounds can influence the complex biochemical processes that take place during the oxidative ageing of wine in barrels, changing the composition of the wine and adding to its stability. The simple extraction of aromatic compounds (volatiles and polyphenols), and tannins from wood can add a richness and complexity to the aroma and taste of wines [107-109].

Generally, aging of wines leads to a loss of the characteristic aromas linked to grape varietal and fermentation, and to the formation of new aromas characteristic of older wines or atypical aromas associated with wine deterioration [110, 111]. Specifically the concentrations of ethyl esters of branched-chain fatty acids are changed during ageing [112], and ageing of wine on the lees was found to decrease the concentrations of volatile compounds imparting a fruity aroma and increasing long-chain alcohols and volatile fatty acids [113]. It is clear that various factors from grape variety, alcoholic and malolactic fermentation to ageing play a role in the final aroma and flavour of a wine. However, the science of wine chemistry – devoted to unravelling the complex matrix of wine aroma – is an exciting and challenging field of study with many new

advances. Some of the problems and procedures involved in detecting aroma compounds in wine will be discussed below.

## 2.6 DETECTION OF WINE AROMA

The previous discussion has only touched upon the complexity of wine aroma. Overall, about 1000 chemical compounds make up the aromatic and flavour profile of a wine [114-116] and some researchers suggested that these profiles can be regarded as footprints or “aromagrams” and can in future be used for identification and quality control purposes [117]. Not only are these aromagrams composed of various chemical classes of compounds as seen above - e.g. alcohols, esters, aldehydes, ketones, acids and sulphur and nitrogen containing compounds - but these compounds have a very wide concentration range in the wine; varying between g/l to the ng/l level [116].

The detection of aroma compounds in wine is therefore a complex undertaking and no single detection method can satisfactorily detect and quantify all these compounds. Wine aroma analysis is also further complicated by its second most abundant component, i.e. ethanol. This compound interferes with most chromatographic analysis methods and therefore it is necessary to concentrate most other odorants, whilst removing most of the ethanol [118]. Thus a wide array of techniques has been developed to extract these compounds from the wine matrix. Some of these include: the simultaneous steam distillation-extraction technique (SDE) [118], stir bar sorptive extraction (SBSE) [119], dichloromethane liquid–liquid extraction followed by concentration under a nitrogen atmosphere (LLE or SE) [120], solid-phase microextraction (SPME) [121, 122], ultrasound extraction [123], solid-phase extraction SPE) [115, 124]. For extraction of only the volatile fraction head-space sampling techniques can be performed. These include headspace solid phase microextraction (HS-SPME) as well as head-space static (HS), head-space dynamic (HD) and purge and trap techniques [116].

To complicate matters even further, some classes of chemical compounds need to be derivatized further before they can be detected. These include dicarbonyl compounds such as glyoxal, methylglyoxal, pentane-2,6-dione, *α*-keto-*γ*-(methylthio)butyric acid and *β*-phenylpyruvic acid - intermediate ketoacid compounds of methional and phenylacetaldehyde [125], as well as aldehydes containing five to eight carbon molecules [126].

Various detection methods also exist and vary in their complexity and ability to detect a wide range of compounds. The type of analysis needed, whether it be qualitative or quantitative would also influence which method to use. It is important to note that no single detection method can be used to measure the entire range of wine aroma compounds. The most common chromatographic technique is gas chromatography, although various types of detection devices may be coupled to it, e.g. gas chromatography equipped with a flame ionisation detector (GC-FID) [123, 127], or a gas chromatograph equipped with a flame photometric detection (GC-FPD) [121, 128]. More advanced systems - able to detect a vast range of compounds – comprise a gas chromatograph coupled to a mass spectrometer (GC/MS) [18, 120, 122, 129]. Various combinations of this coupling exist, e.g. ion trap mass spectrometry (ITMS) after gas-chromatographic analysis [116], or thermal desorption-gas chromatography–mass spectrometry analysis [119]. Non-volatile, or derivitized compounds are sometimes separated and detected by reverse phase high performance liquid chromatography (RP-HPLC) with UV or fluorescence detection [125].

However, it is sometimes necessary for researchers to interact with the analysis of these compounds, by actually smelling them as they are separated from one another. In this case gas chromatography – olfactometry (GC–O) is applied to wine extracts to characterise odour-active zones. In a second run, the aromatic impact of the volatiles identified is evaluated, generally by determining perception thresholds [114]. On the other end of the scale, some researchers are trying to recreate the human nose by constructing so-called “electronic tongues and noses”. This device comprises of an array of non-specific chemical sensors, linked to an advanced pattern recognition technique – mimicking the human olfactory system [130]. However, the

human perception of aroma and flavour cannot be easily mimicked, as it involves a myriad of components, both subjective and objective. Some aspects of human perception will be discussed in the following section.

## 2.7 PERCEPTION OF WINE FLAVOUR AND AROMA

The perception of aroma, i.e. the sense of smell, is also known as olfaction and can occur via two avenues, i.e. orthonasally and retronasally [131]. During orthonasal olfaction, inspired odorant molecules come into contact with the olfactory epithelium and bind to olfactory receptors, leading to a cascade of signal transduction events. Retronasal olfaction is also an important means of detecting odours from food and beverages. While eating or drinking, a small amount of odorant molecules pass through the nasopharynx and into the nose, allowing the appreciation of specific flavours and aromas within the food or beverage. The movements of the tongue and pharynx during chewing and swallowing likely produce this air movement [131]. The retronasal olfactory path also plays an important part in the observation of sweet odours, something of a misnomer as sweetness is normally detected by taste and not by smell. It was found that during the exposure of the mouth to sweetness, the sweet smell in combination with certain odorants that reach the olfactory system via the nasopharynx, caused the olfactory system to associate those odorants with a sweet taste and therefore a smell of sweetness can be observed without tasting something [132].

Odorants from both the orthonasal and retronasal avenues are transported across the olfactory mucus and presented to the olfactory receptors located on the olfactory receptor neurons, from where signal transduction occurs [131]. The olfactory receptors are G protein-coupled receptors (GPCR) - intrinsic membrane proteins with 7 transmembrane (TM) helices, also called 7TM proteins. These receptors also have specific binding sites for antagonists and agonists. Binding of the odorant to its receptor activates a signal transduction cascade involving the cAMP pathway [133]. The signal is further propagated by the influx of $Na^{+}$ and $Ca^{++}$ ions into the cell and further along the axon to the olfactory bulb [131]. Functional magnetic resonance imaging

(fMRI) showed the areas in the brain most involved with olfactory stimulation to be the piriform and orbitofrontal complex as well as the hippocampus, the amygdala, the insular lobe, the cingulate gyrus and the cerebellum [134, 135].

However, the perception of aroma and taste in humans can also be the result of a person's genetic make-up. In studies on the perception of a bitter compound, 6-*n*-propylthiouracil (PROP), it was found that only people with a certain allele at locus 5p15 could detect this compound in aqueous solutions, leading to speculation that a particular person's likes and dislikes regarding food and beverages might in part be due to genetics [136]. Other factors that may affect a person's taste and smell perception are environmental factors such as organic and sulphur-containing air pollutants, as well as surfactants and heavy metals concentrated in the saliva [137]. With regard to the bitterness of wine due to the interaction of salivary proteins and wine tannins it was found that ethanol could increase the perceived astringency of the tannins [138]. Interestingly, taste and aroma were found to be strongly influenced by the visual sense. Descriptions of the "nose" of a wine are dramatically influenced by its colour and fMRI studies have shown a strong response in areas in the brain linked to visual stimuli when the olfactory system was stimulated [139].

From the discussions above it is clear that wine aroma, and aroma in general, is constituted by the complex interactions of several chemical compounds. It is also the case that some of these compounds can further interact with one another in a synergistic or antagonistic effect on the perception of smell and aroma. Studies on tomatoes have shown that addition of sugar to the tomato puree increased the green and musty aromas and decreased floral aroma as well as sour, citrus, and bitter tastes, whilst addition of acids decreased green and floral aromas as well as sweet taste. However, addition of sugar together with acid had the same effect as adding just sugars to the puree [140]. Studies measuring the flavour and aroma of a synthetic strawberry solution and the combination of isoamyl acetate and sucrose found that certain people were insensitive to the synergistic effect of sweetness on aroma and it was postulated that either multisensory convergence – the cross-communication between the taste and aroma modalities,

or learned associations – where a aroma is linked to a taste (and *vice versa*) due to a previous experience – could play a part in this phenomenon [141, 142]. This cross-modality was once again illustrated in a study by Mukai, *et. al* (2007). In order to decrease the bitterness of nutritional products for patients with liver failure, various aromas were combined with the products and it was found that strawberry aroma – evoking sweet and sour pseudo-tastes – could elevate the bitterness threshold in tasters [143].

Sweetness in wine is not normally a role player in determining the final aroma profile, except for natural sweet and dessert wines. However, the two aroma fractions most studied in wine are the fruity (e.g. isoamyl acetate and ethyl butyrate) and woody (e.g. whisky lactone and guaiacol) components. Studies using binary mixtures of these components show that most participants in perception tests were unable to identify both the components, even when their constituents were present in equal amounts [144]. More recent work with the same odour components indicate that that the fruity component can undergo either masking or synergy, depending on the concentration of the woody component. It was also observed that the woody component in fruity–woody mixtures was not perceived in the same way when presented orthonasally and retronasally [145]. Interestingly the non-volatile matrix of the wine also influences the aroma of the wine and it is possible to change this matrix to such an extent that a white wine can smell like a red wine and *vice versa* [146]. Finally, the effect of language and memory on the tasting of wine was also investigated. Experienced wine judges showed superior olfactory recognition when compared to novice judges, but this was not due to enhanced semantic memory and linguistic capabilities for wine-related odours. Rather, it was thought that the superior perception skills of the expert judges protected them from verbal interference, i.e. the need to over-describe a certain odour at the expense of the odour itself [147].

## 2.8 CONCLUSION

It is clear from the above discussion that wine flavour and aroma is a complex interaction of chemical compounds and that differences between wines can be the result of certain varietal

characters present in grapes as odour compounds or precursors that are liberated during the fermentation process. Yeast strains also differ in their ability to produce the full array of chemical compounds that constitute the wine aromatic profile. These compounds can interact with each other and cause synergistic or antagonistic effects. Lastly, it is also important to keep in mind that the appreciation of wine and its aroma bouquet, is subjective and therefore people will also differ on the relative merits or attributes of certain wines. Hopefully there are enough styles, cultivars and wines on the market to satisfy all tastes.

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# Chapter 3

## Research results

### Transcriptome analysis of an industrial yeast strain during a model wine fermentation: Results from oligo-cDNA microarrays

# TRANSCRIPTOME ANALYSIS OF AN INDUSTRIAL YEAST STRAIN DURING A MODEL WINE FERMENTATION: RESULTS FROM OLIGO-cDNA MICROARRAYS

## 3.1 ABSTRACT

Alcoholic fermentation and especially wine fermentation, is one of the most ancient microbiological processes utilized by man, and its biochemistry has been intensely studied. Many questions, however, remain regarding the physiological and molecular adaptations that yeast cells undergo during this process. In the present study, initially undertaken to provide complementary and supporting data to a Serial Analysis of Gene Expression (SAGE) approach, changes in gene transcription during wine fermentation on a genome-wide level are investigated with microarray technology. For this purpose, experimental fermentations were carried out using an industrial wine yeast strain fermenting synthetic grape-must as a model system. Samples for transcriptome analysis were taken at shorter time intervals than any previously reported transcriptome analysis. The fermenting synthetic must was also analyzed for metabolites and aroma compounds important for wine, at the same intervals. Unfortunately some hybridisation problems forced the removal of two of the arrays from the dataset. However, the remaining arrays provide an overview of the transcriptomic changes during a model wine fermentation. The results confirm the temporal gene expression pattern of the fermenting yeast changes reported in previous studies with regard to time and growth stage, and certain functional gene categories showed marked differences in their expression profile. Since the data set analysis short time intervals, genes whose expression show sudden significant up- and down-regulations were further investigated. In addition, the regulation of genes that are linked to aroma production is analysed in more detail.

## 3.2 INTRODUCTION

Alcoholic fermentation is one of the first biotechnological processes utilised by man. There is evidence that the use of alcoholic fermentation by man is at least a few thousand years old [1].

Therefore it is not surprising that the biochemistry of the pathways involved have been closely studied. However, only in the last few years have authors been investigating the effects of alcoholic fermentation on the gene level [2, 3]. Many such studies have been carried out using laboratory yeast strains that differ quite radically from industrial wine yeast strains in their ability to ferment grape must and it has been shown that the gene expression profiles of laboratory and industrial wine yeast strains are also very different for a large section of genes [4].

Alcoholic fermentation in wine is usually performed by industrial species of the yeast *Saccharomyces cerevisiae* [5]. These yeast have evolved and are well adapted to conduct alcoholic fermentation in a high sugar environment in near anaerobic conditions, and in particular to growth in grape-must. During the growth process, the yeast cells are subjected to a wide variety of continuously changing environmental conditions, including many that in the scientific literature would be qualified as stress conditions [6]. These continuously changing conditions will influence gene expression levels in line with required adaptations to the changing environment. Various studies using cDNA microarrays have investigated the effect on the transcriptional level by these stress parameters and/or conditions.

Some major stresses include osmotic stress during rehydration and inoculation [7]. Yin and colleagues [8] studied the effects of both high and low concentrations of glucose on the transcriptional profile of yeast and show that yeast are more sensitive to low glucose levels than previously thought and that different glucose levels provoke different transcriptional responses, highlighting the importance of this compound for yeast metabolism. Another study focussing on the effect of high sugar concentrations on the transcriptome of yeast found that a large set of genes are actively transcribed due to the yeast sensing a high sugar environment [9].

Other stress conditions during fermentation include nutrient limitation and depletion. Grape must usually has low levels of free assimilable nitrogen, in the form of $NH_4^+$ or amino acids, and yeast cells are forced to switch from one source to another, as the preferred forms are depleted [7]. It has been shown that these different nitrogen sources each has a individual effect on the transcript levels of, not only genes related to nitrogen metabolism, but other unrelated genes as well [10, 11]. The combination of high glucose levels and various levels of nitrogen in the fermentation also had striking effects on the gene expression profiles of fermenting yeasts [12]. Addition of supplements such as diammonium phosphate can also influence gene expression [13].

Another very important stress condition encountered during alcoholic fermentation is ethanol toxicity. As sugars are depleted, ethanol accumulates in the yeast cell. Ethanol inhibits growth, viability, transport systems for nutrient uptake and the key glycolytic enzymes [14]. It was also found that a large set of genes was influenced by ethanol shock [14] and growth in the stationary phase in general [15].

Production of white wines and lager beer both may result in cold stress. Lagers are normally fermented at 11 – 14 C, until all the sugars are depleted, when temperatures are lowered to 7 – 8 C [16], whilst white wine fermentations are normally kept at around 13 C [17]. In both cases the lower temperatures had a significant effect on the expression profile, with the wine yeasts fermenting at 13 C showing enhanced transcription of genes involved in the cell cycle, growth control and cellular maintenance when compared to cells fermenting at 25 C [17].

It is thus clear that the yeast cell continuously adapts to a variety of environmental stresses during the course of an alcoholic fermentation. In order to adapt, cells must recognise change, and respond to it by stimulation or inhibition of the production of certain proteins. In order to achieve this, certain genes must be induced or repressed by the yeast. In this study we attempt to elucidate the genes that are induced or repressed due to stress responses during a model wine fermentation.

The most commonly used experimental design used in microarray experiments is the common reference design. In this set-up, all experimental samples are compared to a common reference. In the case of cancer studies this might be normal tissue and the experimental samples; tissues from cancer patients. It is immediately apparent that this design requires a large amount of mRNA from the reference source. Furthermore, such a design requires at least three biological repeats for each time point investigated to be statistically sound. This would have made the present study, which intended to monitor transcript levels at short time intervals, unaffordable. It was thus decided to rather follow a loop design, where each time point is directly compared to its immediate neighbour, as shown in **Figure 3.1**. By its very design the experiment could be used to draw a semi-cumulative picture of gene expression, from time point zero to time point thirteen. By closing the loop by comparing the last time point with the first, the accuracy of the cumulative change can be evaluated. Furthermore, each microarray contains data from the previous time point, as well as the present time point (**Table 3.1**). This experimental design made it possible to investigate real-time adjustments of the transcriptome in response to rapidly changing conditions during the model wine fermentation. Other studies on the transcriptome of wine yeasts either used a maximum of six time points during the whole course of the fermentation [3], or relied on a chemostat to obtain a steady state [11], or focussed on a particular growth stage or fermentation condition [9, 12, 13]. This study is therefore unique and should help elucidate some of the small-scale changes occurring in the transcriptome of an industrial yeast strain during a model wine fermentation. In order to be representative and reproducible, fermentations were carried out in synthetic must MS300 using the globally and commonly used industrial wine yeast strain EC1118. The data show that the largest number of genes involved in cellular stress responses are expressed during the latter stages of the fermentation, when nutrient limitation is severe and ethanol toxicity becomes a problem for the yeast cells. Analysis of rapid temporal changes in the transcriptome, i.e. spike analysis, reveals that a large set of genes involved in the machinery needed to quickly transcribe, translate and process any proteins needed to fully take advantage of any environmental condition that might arise are

subject to this phenomenon. However, analysis of genes that impact on the production of aroma compounds show no such spikes and are expressed at constant levels through-out the fermentation, indicating that aroma compound formation occurs gradually from the start of the fermentation.

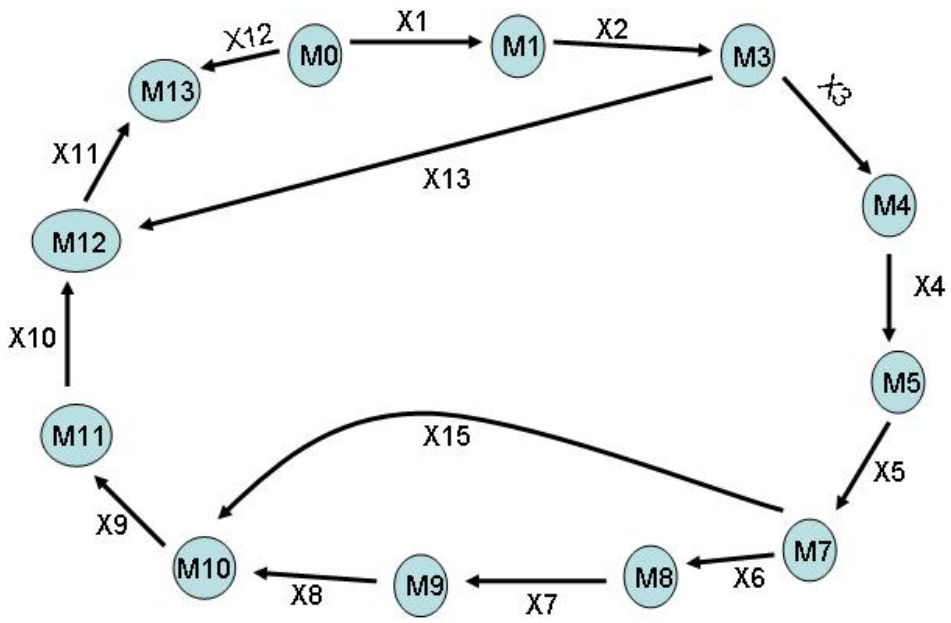


**Fig. 3.1**. Experimental loop design for the current set of microarray experiments. The shaded circles refer to the actual time points (see Table 3.1 for details); whilst X1 to X15 refer to the actual microarrays themselves. There is no microarray X14; this was because X7 was repeated during the experiment. X13 and X15 are referred to as cross-references and can be used for data validation.

## 3.3 MATERIALS AND METHODS

### 3.3.1 GROWTH AND MEDIUM CONDITIONS

The industrial wine yeast EC1118 (Lallemand) and the defined synthetic wine fermentation medium MS300 was used for this study. The medium was adapted from Bely *et al.* [18] and contained the

following: 150 g/l glucose, 150 g/l fructose, mineral salts (750 mg/l $KH_2PO_4$, 500 mg/l $K_2SO_4$, 250 mg/l $MgSO_4$.7H2O, 155 mg/l $CaCl_2.2H_2O$, 200 mg/l NaCl, 4 mg/l $MnSO_4.H_2O$, 4 mg/l $ZnSO_4$, 1 mg/l $CuSO_4.5H_2O$, 1 mg/l KI, 0.4 mg/l $CoCl_2$.6H2O, 1 mg/l $H_3BO_3$, 1 mg/l $NaMoO_4.2H_2O$), vitamins (20 mg/l myo-inositol, 2 mg/l nicotinic acid, 1.5 mg/l calcium panthothenate, 0.25 mg/l thiamine HCl, 0.25 mg/l pyridoxine HCl, 0.003 mg/l biotin), 300 mg/l of assimilable nitrogen provided by a mixture of 19 amino acids (612.6 mg/l L-proline, 505.3 mg/l L-glutamine, 374.4 mg/l L-arginine, 179.3 mg/l L-tryptophan, 145.3 mg/l L-alanine, 120.4 mg/l L-glutamic acid, 78.5 mg/l L-serine, 759.2 mg/l L-threonine, 48.4 mg/l L-leucine, 44.5 mg/l L-aspartic acid, 44.5 mg/l L-valine, 37.9 mg/l L-phenylalanine, 32.7 mg/l L-isoleucine, 32.7 mg/l L-histidine, 31.4 mg/l L-methionine, 18.3 mg/l L-tyrosine, 18.3 mg/l L-glycine, 17.0 mg/l L-lysine, and 13.1 mg/l L-cysteine) corresponding to 180 mg nitrogen and 460 mg/l ammonium chloride (corresponding to 120 mg nitrogen). The medium contained anaerobic factors (15 mg/l ergosterol; 5 mg/l sodium oleate), added to the medium in 1 ml Tween 80/ethanol (50/50, v/v). The pH was buffered at 3.3 with NaOH. Strains were grown overnight in 5 ml of YPD medium and inoculated into the sample pre-culture. Sample pre-cultures consisted of 50 ml MS300 medium in 250 ml Erlyn-Meyer flasks and were placed on a shaking incubator at 30 ºC for overnight growth. Strains were then inoculated into the chemostat containing 10 litres of MS300 medium, so that the total amount of cells after inoculation in the chemostat would be 2 x $10^6$ cells per litre. The chemostat was then flushed with nitrogen gas to ensure complete anaerobic growth conditions.

### 3.3.2 SAMPLING

Samples were taken at six hour intervals during the first 40 hours of the fermentation and thereafter at 12 to 24 hour intervals. Samples were centrifuged and the supernatant was used for the analysis of metabolites and fermentation parameters, whilst the cell pellet was washed with 0.9% (w/v) NaCl solution and stored at -80 °C until RNA isolation. The sampling time points during the model wine fermentation that were used for the microarray experiments are shown in **Table 3.1**.

**Table 3.1**: The following time points during the model wine fermentation were used for the microarray experiments

| Name | Time |
|---|---|
| M0 | 0 hr |
| M1 | 6 hr |
| M3 | 18 hr |
| M4 | 24 hr |
| M5 | 30 hr |
| M7 | 42 hr |
| M8 | 60 hr |
| M9 | 72 hr |
| M10 | 108 hr |
| M11 | 116 hr |
| M12 | 138 hr |
| M13 | 164 hr |

### 3.3.3 MEASUREMENT OF METABOLITES AND FERMENTATION PARAMETERS

Clarified supernatant was subjected to various tests to determine the levels of metabolites produced or consumed during the model wine fermentation. The ammonia concentration was determined by using an enzymatic kit, whilst the levels of free available nitrogen (FAN) – this includes all nitrogen available for metabolism with the exception of ammonia and proline – was measured using the NOPA procedure [19]. Concentrations of glucose, fructose, ethanol and a variety of other organic acids were determined by High Performance Liquid Chromatography (HPLC) analysis. These results were confirmed by the WineScan FT 120 instrument (Foss, Denmark) that employs a Michelson interferometer to generate the FT-IR (Fourier infra-red) spectra. The samples (30 ml) were pumped through the $CaF_2$-lined cuvette housed in the heater unit of the instrument. Thus the temperature of the samples was brought to exactly 40ºC before analysis. Samples were scanned from 5011-929 $cm^{-1}$ at 4 $cm^{-1}$ intervals, which includes a small section of the near infra-red (NIR) spectrum. The frequencies of the NIR beam transmitted by a sample were recorded at the detector and used to generate an interferogram. This interferogram comprised of a series of 10 scans being processed by Fourier transformation and corrected for the

background absorbance of water to generate a single beam transmittance spectrum. Two transmittance spectra for each sample were generated in order to calculate the absolute repeatability of the spectral measurements. This was in accordance with the manufacturers guidelines (WineScan FT120 Type 77110 and 77310 reference Manual, Foss, Denmark, 2001). The transmittance spectra were finally converted into linearised absorbance spectra through a series of mathematical procedures.

### 3.3.4 RNA ISOLATION AND MANIPULATION AND cDNA LABELLING

Standard laboratory practices were implemented when working with RNA [20]. Total RNA was isolated from the yeast pellets with the Bio101 RNA Extraction Kit (BIO 101, Inc., Vista, CA, USA). The RNA was spectrophotometrically quantified and visualised for quality by agarose gel electrophoreses. Total RNA samples were stored at -80 °C until needed for microarray analysis. The total RNA underwent DNase (RNase free) treatment in order to degrade any contaminating genomic DNA fragments. Total RNA was incubated at 37 ºC for 15 minutes in the presence of the RNase free DNase, after which the samples treated with a phenol chloroform mix, followed by ethanol precipitation in order to obtain pure messenger RNA (mRNA). cDNA was synthesised from the mRNA, using the Superscript III Reverse transcriptase system (Invitrogen, Carlsbad, CA, USA) and a special RT primer oligo from either the Cy3 or Cy5 Genisphere kits (Genisphere LLC, Hatfield, PA, USA). The cDNA and fluorescent 3DNA (Cy3 (green) or Cy5 (red)) reagent were then hybridised to the yeast microarray glass slides. The 3DNA fluorescent reagent hybridises to the cDNA due the inclusion of a “capture sequence” that is complementary to a sequence on the 5’ end of the RT primer. This is known as indirect labelling.

### 3.3.5 MICROARRAY ANALYSIS

Microarray slides were obtained from The University Health Network, Toronto Canada (www.microarray.ca). The Yeast 6.4K Array (Y6.4K) is a double-spotted array containing 6,240 yeast ORFs (plus control spots totalling 6.4K). After hybridisation overnight, the slides were washed

and scanned by laser in the green and red wavelengths. The overlay of the two channels was then used to determine the ratio of red to green. Background subtraction was performed by the Genepix program, using the local area algorithm – calculating the mean background value from the area around each spot and subtracting it from the red and green values for that spot - and the results exported as numerical data in the form of gpr files. The online software suite GEPAS (http://gepas.bioinfo.cipf.es/) was then used to normalise the data by implementing the print tip loess algorithm. Microarray visualization and analysis software from The Institute for Genomic Research (TIGR), TMev, was used to visualise the data and to cluster genes with similar expression profiles.

### 3.3.6 STATISTICAL METHODS FOR EXTRACTING VALUE FROM THE DATASET

#### 3.3.6.1 NORMALIZATION AND QUALITY ASSESSMENT

The Limma R package [21] was used to do background correction [22] and quantile normalization [23] across arrays was done for the red and green channels separately. Expression data was subsequently extracted from both the red and the green channels and stored separately. Background plots were inspected and two microarrays removed due to what appear to be hybridization/washing issues that led to significant spatial anomalies. However, there were significant spatial anomalies evident on most microarrays so further analysis was done as follows.

#### 3.3.6.2 AUTO-CORRELATION

A perl programme was written to restructure the Limma output files so that sequential time course vectors were created for each probe. A new set of vectors were subsequently created for probes in both the green and red channels which contained time points that were common to both channels.

36 Million Pearson Correlations for each possible probe pair from both channels were generated with mcxarray and converted to a pairwise format with mcxdump [24].

A Perl program was written to select probes if their green and red channel vectors had a Pearson correlation coefficient > 0.7. This reduces the number of reliable probes extracted from the microarrays to 1439. This auto-correlated set of 1439 probes was used for the rest of the analysis.

#### 3.3.6.3 SPIKE DETECTION

An algorithm was designed to detect short spikes in up or down regulation of expression and implemented in Perl. The algorithm uses a sliding window to traverse the time course of each and every probe in sets of three time points in both the red and the green channel vectors. The algorithm creates a ratio between each contiguous time points and selects probes which show a minimum of a two fold increase or decrease between the first and second time points and a subsequent minimum of a twofold decrease or increase between the second and third time points. The programme reports both the probe id and full time course expression vector from both the green and the red channel of each probe that meets these criteria.

#### 3.3.6.4 CLUSTER ANALYSIS

The time course expression vectors in which a spike had been detected were analyzed further with hierarchical clustering [25]. A Perl programme was written to create an annotated graph structure (including orf_id, gene names, descriptions and GO terms from SGD) from the resulting clusters, only reporting connected clades that met a Pearson distance threshold of < 0.2 (the equivalent of a Pearson correlation threshold of > 0.8). The resulting annotated graph structure was visualized in Cytoscape [26] for further biological interpretation.

## 3.4 RESULTS AND DISCUSSION

### 3.4.1 GENERAL OVERVIEW OF THE DATASET

The aim of this study was to investigate the genome wide gene expression pattern of an industrial wine yeast during a wine fermentation at short time intervals. To ensure reproducibility of the model fermentations, three separate 10 litre fermentations were carried out and the results obtained in respect to time of fermentation, concentration of ethanol produced, rate of sugar metabolism and final concentrations of various other secondary metabolites were compared and found to be sufficiently similar to indeed satisfy the requirements for reproducibility. The fermentation parameters of the fermentation used for the microarray data generation can be seen in **Figure 3.2** and clearly indicate that the fermentation was completed, i.e. that all sugars had been metabolised, and that growth kinetics and production of secondary metabolites was as in the control fermentations. The 12 sampling points – situated through-out the course of the fermentation - were compared directly to one another, in a loop-like fashion (as shown in **Figure 3.1**).

Analysis of background signals present on each of the microarrays revealed hybridisation anomalies, especially in arrays X6 and X7. Normally, hybridisation problems would manifest themselves equivalently in both channels, i.e. hybridisation patterns would look alike in both the red and green channel. However, this is not the case with arrays X6 and X7 as can clearly be seen from **Figure 3.3** (only array X7 is shown, panel A indicates the green channel and panel B, the red channel). Multivariate data analysis further confirmed these physical phenomena by indicating that whilst the rest of the arrays were spread out across the horizontal axis in a Principal Component

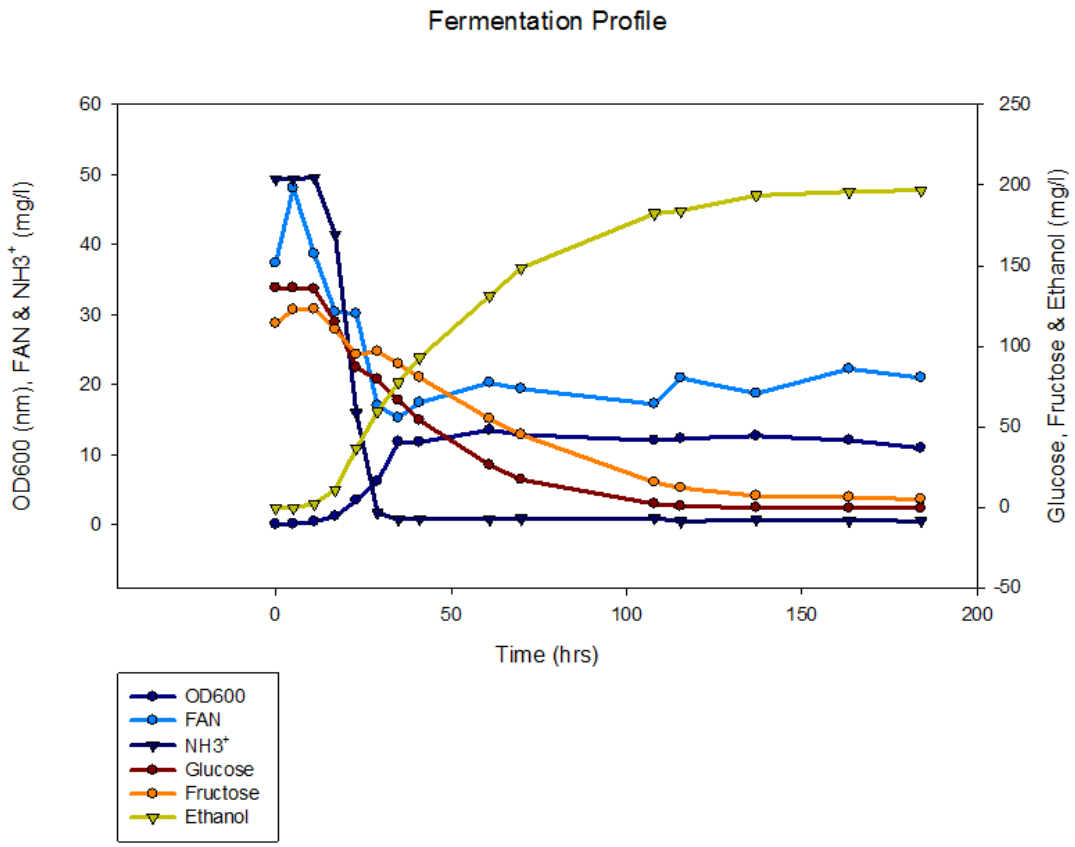


**Fig. 3.2**. Fermentation profile of the industrial wine yeast EC1118 in a synthetic grape must. OD600 represents the optical density of the culture media at 600 nm, thus is an indication of the number of yeast cells present in the media at a specific time. An OD600 value of 0.1 is equal to 3 x $10^6$ cells per ml. NH3 represents the concentration of $NH3^+$ ions in the media, added as $NH_4Cl$. FAN represents the concentration of free assimilable nitrogen compounds in the media, with the exception of $NH_4Cl$.

Analysis plot (**Figure 3.3**, panel C) – a putative indication of the expected variance due to biological differences over the time course of fermentation – arrays X6 and X7 were complete outliers in both the red and green channels (even thought the red and green channels represent different time points on these arrays). Therefore it was decided to exclude arrays X6 and X7 from the study. Unfortunately this means that a gap exists between hours 42 and 72 of the model wine fermentation.

It is clear from **Table 3.2** that there are differences in the number of genes induced or repressed between the different microarrays. It would seem that between 0 and 6 hours (i.e. array X1) only a small percentage of the 6218 genes' expression levels are changed, 3.3% of all genes are induced, while almost 4% are repressed. **Table 3.2** also shows that a greater percentage of genes expression patterns are altered in array X2, compared to array X1. Altogether 519 or 8.35% of genes are up-regulated, but only 58 genes or 0.93% is repressed. In array X3, only about 2.8% of the genes are induced compared to the 4.3% that are repressed.

These three microarrays contain time points corresponding to the early growth phase. From the composition of induced and repressed genes in these three arrays it would seem that the large number of up-regulated genes involved in transcription, protein synthesis, glycosylation and transport, show a clear adaptive response of the yeast cells to their new environment. Only few of the glycolytic genes are strongly induced. This may be because these genes are constitutively expressed at high levels, so that only minor inductions may be necessary to adapt to the new environment with its high concentration of glucose and fructose.

The yeast cells have entered the logarithmic growth at the time point corresponding to microarray X4, and the same genes continue to be induced or repressed as in the first few arrays. A major shift in transcription occurs in the period between array X4 and X5, since a large number of genes are induced (12.7%) and repressed (7.5%) compared over a period of 12 hours. In particular, genes encoding for proteins involved in the metabolism of amino acids, pyrimidine, glycogen, fatty acids, fructose, sphingolipids, sterols, heme, trehalose, phospholipids, as well as ethanol utilisation are induced. This suggests that a major metabolic adaptation has occurred at this stage of fermentation.

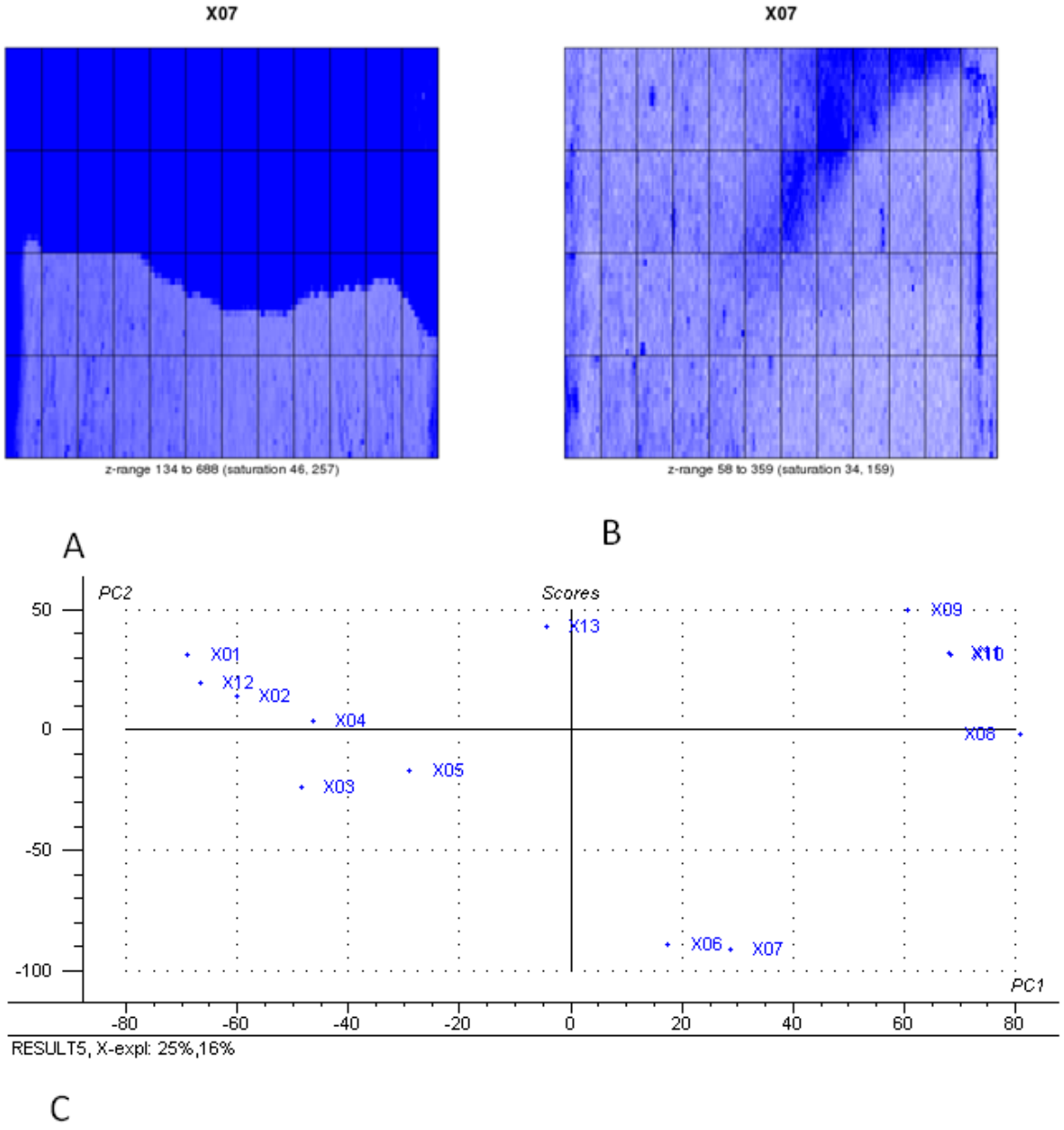


**Fig. 3.3**. Illustration of hybridisation problems encountered with some of the microarrays. Panels A and B show the background hybridisation of array X07, in both the green and red channels, respectively. Panel C indicates the variance between the arrays as visualised by principle component analysis.

**Table 3.2**: Number of genes repressed or induced for different microarrays (6218 genes in total)

| Array name | Range (hours) | Genes induced | Genes repressed |
|---|---|---|---|
| X1 | 0 – 6 | 204 | 248 |
| X2 | 6 – 18 | 519 | 58 |
| X3 | 18 – 24 | 175 | 269 |
| X4 | 24 – 30 | 135 | 80 |
| X5 | 30 – 42 | 789 | 465 |
| X6* | 42 – 60 | 396 | 149 |
| X7* | 60 – 72 | 669 | 283 |
| X8 | 72 – 108 | 589 | 229 |
| X9 | 108 – 116 | 575 | 237 |
| X10 | 116 -138 | 543 | 265 |
| X11 | 138 -162 | 712 | 398 |
| X12 | 0 – 162 | 815 | 1075 |
| X13 | 18 – 138 | 539 | 60 |
| X15 | 42 - 108 | 547 | 199 |

*Arrays X6 and X7 were excluded from analysis due to hybridisation problems and are only included in this table in order to view the number of genes induced and repressed throughout the complete dataset

The expression of genes in arrays X8, X9 and X10 are very similar and it seems that the yeast cells have entered a steady state. Interestingly, a large number of genes involved in protein synthesis are still being induced during this stationary growth phase, indicating that the cells are still metabolically active. Also consistent with the latter stages of an alcoholic fermentation is the induction of various genes involved in the stress response of the yeast cell. It is also notable that during the last two time points, i.e. from 138 hours to 164 hours, a relatively large amount of genes are induced (almost 12%) as well as repressed (almost 7.5%) in relation to the previous three arrays.

Array X12, which compares time point 0 with time point 12, as expected, shows the largest number of genes induced or repressed, since it represents the cumulative changes in gene expression

levels observed in the individual arrays described above. Of the total of 6218 genes, 815 or 13% are induced and 1075 or 17% are repressed. It is immediately striking that a proportionally larger number of genes are repressed. This is expected, since the last array represents the end of fermentation and many metabolic functions are being reduced. Furthermore a study investigating various DNA repair enzymes found that the mRNAs experienced drastic increments in their stabilities in response to gradual depletion of essential nutrients. These mRNA stability profiles differed widely, suggesting a dynamic modulation rather than a passive process. Although these genes were much more frequently transcribed during the fermentative growth than in the stationary phase, this could be explained by equating low expression levels with increased mRNA stabilities [27]. It is thus possible to explain the reduced gene expression for some genes with increased mRNA stability mechanisms in response to changes in nutrient-availability.

Many of the up-regulated genes are involved in transcription, mRNA transport –processing, -splicing, as well as, protein synthesis, -folding, -glycosylation, -degradation and –transport. As expected a large number of these genes will also be involved in the metabolism and/or biosynthesis of various compounds, such as, maltose, fatty acids, ethanol, sterols, amino acids, acetyl-CoA, trehalose, glycerol, glycogen, phosphate, galactose, sterols, sulphate, flavin, phospholipids, mannose, heme and nitrogen. Many of the up-regulated genes are involved in functions such as mitochondrial biogenesis, DNA repair, tRNA maintenance, cell cycle, cell wall biogenesis, chromosome maintenance, cytoskeleton, mitosis, cell size, signalling, bud emergence, as well as various stress responses, e.g. *YBR016W*, *SSE2*, *PPZ2*, *ASK10*, *HYR1*, *PPZ1*, *LYS7*, *PAI3* and *ATX2*. Numerous genes involved in glycolysis and catabolite repression are also amongst the genes induced, including: *CCR4*, *GIP1*, *PDB1*, *GPM2*, *GAL83*, *GIP2*, *ENO2*, *PIG2*, *FBA1*, *GPM1*, *SDS22*, *PDC5*, *PGM2* and *STO1*.

It is natural that many genes of the same functional category are also repressed as these categories do not provide a qualitative picture, i.e. a protein involved in protein synthesis will be

included in the same category as a protein acting as a protein synthesis repressor. Many genes are involved in transcription, protein synthesis, -glycosylation, -transport, -targeting, -folding and –degradation. Genes involved in the metabolism and/or biosynthesis of pyrimidine, various amino acids, sterols, fructose, purine, acetate ester, isoprenoid, fatty acids and glycogen are down-regulated. This is distinctly less than the metabolism genes that are up-regulated. A few of the glycolytic genes are also repressed, i.e. *GCR2*, *GPM3*, *TYE7* and *GCR1*. The majority of the down-regulated genes, however, encode for products involved in the cell cycle, cell size, cytoskeleton, meiosis, DNA replication and repair, chromatin structure, endocytosis and resistance to various factors. It is once again important to stress that these changes represent genes regulated over a very long period of time and does not take smaller, temporal and transient changes in gene expression into account.

### 3.4.2 FUNCTIONAL CATEGORISATION OF THE MICROARRAY DATASET

In order to simplify the analysis of the microarray data, genes were separated according to their functional capacity, according to the MIPS *Saccharomyces cerevisiae* functional catalogue (http://mips.gsf.de/proj/yeast/catalogues/funcat/). The genome of *Saccharomyces cerevisiae* was thus divided into 19 categories. It is important to note that in some cases a gene might be included in more than one functional category if its gene product is involved in more than one cellular process. **Table 3.3** shows the 19 functional categories, as well as the number of genes in each category. The last two columns also show the number of genes, of a specific category, induced or repressed in the different microarrays.

During the course of the model wine fermentation three distinctive growth phases can be observed. The initial 24 hours of the fermentation is called the early growth phase (arrays X1 to X3), followed by the logarithmic growth phase (arrays X4 and X5) that continues until about 72 hours. Thereafter yeast cells enter the so-called the stationary phase until the fermentation is complete (arrays X8 to X11). It is perhaps easier to visualise the above results in the form of a bubble-chart, combining

arrays from the different stages of the model wine fermentation. **Figure 3.4** represent the percentages of genes induced (red bubbles) and repressed (green bubbles) during the early growth, logarithmic growth and stationary phase of a model wine fermentation, respectively. The size of the bubble indicates the percentage of the genome that the specific functional category represents.

**Table 3.3**. *Saccharomyces cerevisiae* functional catalogue and the number of genes regulated in each category in each microarray

| Function | Nuber of genes | Upregulated | | | | | | | | | | | | | | Downregulated | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | X11 | X12 | X13 | X15 | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | X11 | X12 | X13 | X15 |
| Metabolism | 808 | 19 | 26 | 25 | 13 | 83 | 51 | 86 | 54 | 56 | 55 | 54 | 84 | 52 | 50 | 46 | 6 | 22 | 3 | 42 | 7 | 37 | 5 | 3 | 3 | 9 | 611 | 1 | 22 |
| Energy | 190 | 7 | 7 | 6 | 0 | 12 | 9 | 25 | 13 | 13 | 14 | 14 | 21 | 11 | 19 | 13 | 2 | 2 | 0 | 9 | 0 | 2 | 1 | 0 | 1 | 0 | 45 | 1 | 6 |
| Cell Cycle/DNA processing | 476 | 10 | 21 | 12 | 9 | 50 | 35 | 54 | 44 | 43 | 35 | 42 | 65 | 34 | 45 | 21 | 3 | 16 | 0 | 23 | 1 | 22 | 3 | 2 | 1 | 4 | 102 | 1 | 14 |
| Transcription | 547 | 16 | 24 | 22 | 16 | 59 | 35 | 63 | 41 | 34 | 46 | 40 | 64 | 39 | 42 | 20 | 3 | 14 | 0 | 27 | 4 | 21 | 2 | 0 | 3 | 3 | 130 | 0 | 14 |
| Protein Synthesis | 268 | 6 | 7 | 9 | 5 | 31 | 17 | 32 | 33 | 26 | 25 | 23 | 26 | 10 | 32 | 12 | 3 | 8 | 0 | 12 | 2 | 0 | 1 | 0 | 0 | 1 | 68 | 0 | 6 |
| Protein Fate | 461 | 8 | 10 | 21 | 11 | 31 | 29 | 52 | 40 | 44 | 38 | 38 | 57 | 30 | 38 | 15 | 4 | 15 | 1 | 25 | 4 | 26 | 6 | 1 | 4 | 5 | 112 | 2 | 12 |
| Cellular Transport | 357 | 11 | 8 | 12 | 4 | 31 | 23 | 29 | 27 | 25 | 33 | 30 | 47 | 20 | 21 | 14 | 2 | 110 | 0 | 17 | 3 | 18 | 3 | 1 | 0 | 2 | 71 | 0 | 8 |
| Cellular Communications/ Signal Transduction | 45 | 1 | 1 | 1 | 0 | 3 | 3 | 3 | 2 | 4 | 4 | 7 | 6 | 2 | 4 | 1 | 1 | 2 | 0 | 4 | 0 | 4 | 1 | 0 | 1 | 0 | 10 | 0 | 4 |
| Cell Rescue, Defence & Virulence | 201 | 4 | 8 | 3 | 2 | 25 | 12 | 21 | 19 | 12 | 17 | 18 | 26 | 15 | 24 | 12 | 3 | 4 | 0 | 16 | 2 | 11 | 3 | 1 | 1 | 3 | 43 | 1 | 8 |
| Regulation of/ interaction with Cellular Environment | 148 | 1 | 4 | 5 | 1 | 16 | 9 | 14 | 8 | 10 | 18 | 16 | 16 | 11 | 17 | 4 | 1 | 2 | 0 | 10 | 3 | 12 | 0 | 1 | 0 | 1 | 30 | 0 | 3 |
| Cell Fate | 320 | 8 | 14 | 9 | 8 | 23 | 20 | 28 | 22 | 22 | 16 | 22 | 42 | 22 | 20 | 13 | 5 | 11 | 0 | 19 | 2 | 21 | 2 | 2 | 1 | 3 | 66 | 0 | 11 |
| Transposable Elements, etc | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Control of Cellular Organization | 144 | 4 | 8 | 1 | 3 | 13 | 13 | 15 | 11 | 12 | 14 | 8 | 20 | 12 | 14 | 4 | 1 | 5 | 0 | 5 | 2 | 9 | 0 | 0 | 0 | 0 | 33 | 0 | 10 |
| Sub-cellular Localisation | 1661 | 42 | 57 | 51 | 31 | 159 | 105 | 172 | 136 | 121 | 129 | 128 | 192 | 103 | 133 | 70 | 16 | 47 | 3 | 88 | 11 | 70 | 12 | 3 | 8 | 13 | 372 | 2 | 48 |
| Protein Activity Regulation | 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Protein with binding function | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Transport facilitation | 222 | 3 | 5 | 7 | 2 | 26 | 7 | 20 | 13 | 19 | 23 | 19 | 27 | 8 | 17 | 9 | 2 | 4 | 0 | 9 | 2 | 8 | 1 | 0 | 0 | 0 | 50 | 2 | 6 |
| Classification not clear | 84 | 4 | 0 | 5 | 1 | 7 | 7 | 8 | 3 | 11 | 6 | 6 | 12 | 4 | 6 | 6 | 7 | 1 | 3 | 0 | 5 | 2 | 6 | 0 | 0 | 0 | 21 | 0 | 2 |
| Unclassified proteins | 1727 | 52 | 69 | 58 | 34 | 190 | 114 | 208 | 159 | 178 | 153 | 165 | 187 | 123 | 178 | 68 | 13 | 50 | 2 | 92 | 7 | 81 | 3 | 2 | 3 | 15 | 397 | 2 | 36 |

It is clear from **Figure 3.4** that the early growth phase differs quite dramatically from the latter two growth phases. Genes falling within the functional category “cellular transport” seem to be heavily repressed during the early growth phase. However, as the size of the bubble indicates, this category comprises only a relatively small number of genes and thus the results can be a bit misleading as the repression of a few genes will be seen as a large percentage.

In the logarithmic growth phase (panel B) one can clearly see that the percentages of genes up-regulated are larger than that of the down-regulated genes (6 – 8% *vs*. 2 – 4%). An even clearer separation is seen during stationary phase (panel C). During this late stage of the fermentation a very small number of genes are repressed (0 – 2%), whilst a relatively larger number of genes from the same functional categories are up-regulated (6 – 10%). This follows the trend observed in **Table 3.2**, that indicated that overall during all time points, more genes were induced than were repressed.

However, the above scenario involves averaging of close lying time points and this subjective process might obscure some interesting findings. Therefore it is of more value to look at individual arrays as well. It is clear from **Table 3.3** that there is a general trend in the induction and repression of genes in the 19 functional categories during the fermentation period. In most of the functional categories array X1 has the smallest number of genes induced or repressed, although the number of repressed genes are uniformly higher than those induced. It is important to note that in array X1, time points 0 and 6 hours are compared. Some of the genes that are induced include ones whose gene products are involved in protein synthesis, glycosylation as well as degradation. Some others include proteins for cell wall biogenesis, cell surface assembly and flocculation (*MUC1*). Only one gene involved in glycolysis, *TDH2*, is induced. Of course a large number of unknown genes are also induced. Among the repressed genes are quite of number of genes encoding for proteins involved in metabolism of compounds other than glucose or fructose, e.g. methionine-, sterol-, trehalose-, lysine-, arginine-, histidine-, pyrimidine-, glycogen-, and fatty acid metabolism. Strangely enough the genes *GPM1*, *GPM2* and *FBA1*, all involved in glycolysis are also repressed. Once again a large number of the genes are classified as having unknown gene products.

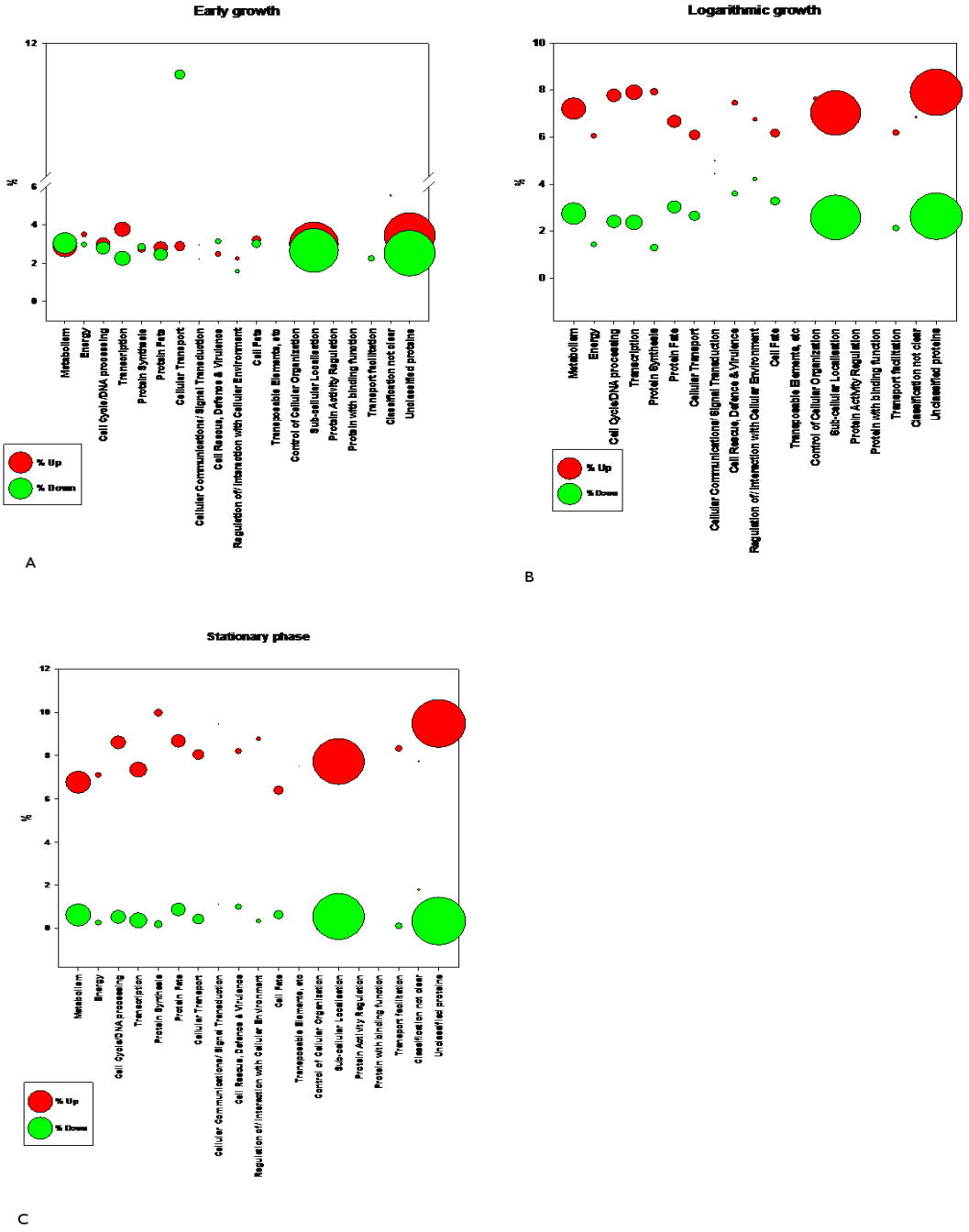


**Fig. 3.4**. Bubble-charts, combining arrays from the early growth phase (arrays X1 to X3) – panel A, the logarithmic growth phase (arrays X4 and X5) – panel B and the stationary phase (arrays X8 to X11) – panel C, observed during the model wine fermentation. The figure indicates the percentages of genes in each functional category, induced (red bubbles) and repressed (green bubbles) during the early growth phase. The size of the bubble indicates the percentage of the genome that the specific functional category represents.

During arrays X2 and X3, the number of regulated genes is increased, with more genes being induced than repressed. In the latter category we find genes involved in amino acid biosynthesis, glycogen metabolism, protein synthesis and transcription, the cell cycle, cytoskeleton assembly, meiosis and various metabolism pathways. Of the up-regulated genes, some are involved in intracellular signalling, transport (in this case *HXT16*), cell wall biogenesis, protein glycosylation as well as flocculation and pseudohyphal growth i.e. *MUC1*, *STE12*, *MSS11* and *DFG5*. Interestingly many genes are also involved in signalling, transcription, protein synthesis and processing, secretion, cell size, mitosis and various stress responses.

It is clear that array X5 represents a major regulatory period for the yeast cell. In almost all of the functional categories, a dramatic increase in the number of genes induced or repressed can be seen. Induced genes include those for protein synthesis and a number of the glycolysis genes, i.e. *PGI1*, *LAT1*, *GPM3* and *PYK2*. Some of the repressed gene include *REG2* and *PIG2*; involved in glucose repression, a number of genes involved in the cell cycle and cytoskeleton, as well as the TCA cycle and oxidative stress response. Two glycolytic genes, *PFK2* and *ADH4* are also repressed. From **Figure 3.1** it is clear that almost all of the available nitrogen has been taken up by the yeast cells at this stage and thus one of the nitrogen catabolism genes, *DAL80* is also repressed at this time point. Such dramatic changes in the growth medium will obviously greatly influence the gene expression profile of the yeast.

Arrays X8 to X11 represent yeast cells in the stationary phase. Consistent with the latter stages of an alcoholic fermentation is the induction of various genes involved in the stress response of the yeast cell. Many genes involved in transcription, mRNA transport and protein synthesis are strongly repressed. It would also seem as if the cells are at some kind of metabolic hiatus at this stage, because uniformly many metabolic genes, which were up regulated in the previous arrays, are now

being repressed. This may also be due to the depletion of various nutrients in the medium at this stage, as seen in **Figure 3.2**.

At the late stage in fermentation, with an almost total depletion in nutrients and a high ethanol concentration, the cells are expected to undergo a switch from an actively metabolising state to an inactive state. In line with this expectation, the majority of repressed genes are categorised as biosynthesis and metabolism (including glycolysis), as well as cell cycle, mRNA processing, transcription and protein synthesis and -targeting. Inversely, however genes involved in cell wall organisation and biogenesis, and cytoskeleton are amongst those that are induced. This might be a protective measure in these unfavourable conditions. It might be helpful to keep in mind at this stage that these results only reflect the relative levels of mRNA in the cells from one time point to the next, and that there might be differences in the actual protein levels, due to mRNA stability and post translational modifications.

### 3.4.3 INVESTIGATION OF KEY INFLUENCES ON THE TRANSCRIPTOME OF AN INDUSTRIAL WINE YEAST DURING A MODEL WINE FERMENTATION

In the above section the influence of the nutritional state of the medium on the regulation of some genes was touched on. However, it is worthwhile to make a thorough examination of the model fermentation, identifying key moments and determining if these had any effect on the transcriptome of the industrial wine yeast strain. From **Figure 3.2** it is possible to determine that the early growth or lag phase lasted form 0 hours to about 20 hours, encompassing arrays X1, X2 and X3. The logarithmic growth phase lasted from 20 hours to about 35 hours and is represented by arrays X4 and X5. The last phase of the fermentation is called the stationary phase, in which cells are metabolically active, but no longer grow and divide. Arrays X8 to X11 represent these conditions. Medium composition and nutrient availability also changes dramatically throughout the model fermentation. The free assimilable nitrogen component is at 50% at about 20 hours and completely depleted after about 35 hours. Glucose is preferentially consumed with regards to fructose with

50% depletion between 35-40 hours for glucose and 50 hours for fructose. The latter is completely depleted at about 137 hours compared to the 108 hours for glucose. Ethanol is produced during the fermentation and reaches its 50% concentration level of about 90 g/l at 40 hours with the maximum level of 196.96 g/l reached after 137 hours. This equates to a final ethanol percentage of 15.56 % (w/v).

The data set was re-analyzed to identify patterns in transcriptional regulation using K-Means/Median clustering. This technique involves the random assignment of genes into a chosen number of clusters, calculating the mean/median expression profile of each cluster and shuffling genes among clusters such that each gene is now in the cluster whose mean expression profile is the closest to that gene's expression profile. These steps are repeated until genes cannot be shuffled around anymore or a user-specified number of iterations has been reached. **Figure 3.5** represents the 20 clusters obtained as well as each clusters mean/median expression profile and the number of genes comprising each cluster.

Although these clusters are meaningless as such, it gives a clear indication of possible expression patterns amongst the genes of the yeast strain and as such is useful in linking transcriptome to external factors such as fermentation parameters discussed above, as well as identifying genes that are co-expressed. Several different expression patterns can be seen amongst the clusters in **Figure 3.5**. The largest number of genes show no increase or decrease in transcription through-out the fermentation period, however there are clusters that show sudden increases in transcriptional regulation in the early stages of the fermentation (black cluster), the middle (purple cluster) and the late stages of the fermentation (blue cluster), as well as those increasing during the fermentation

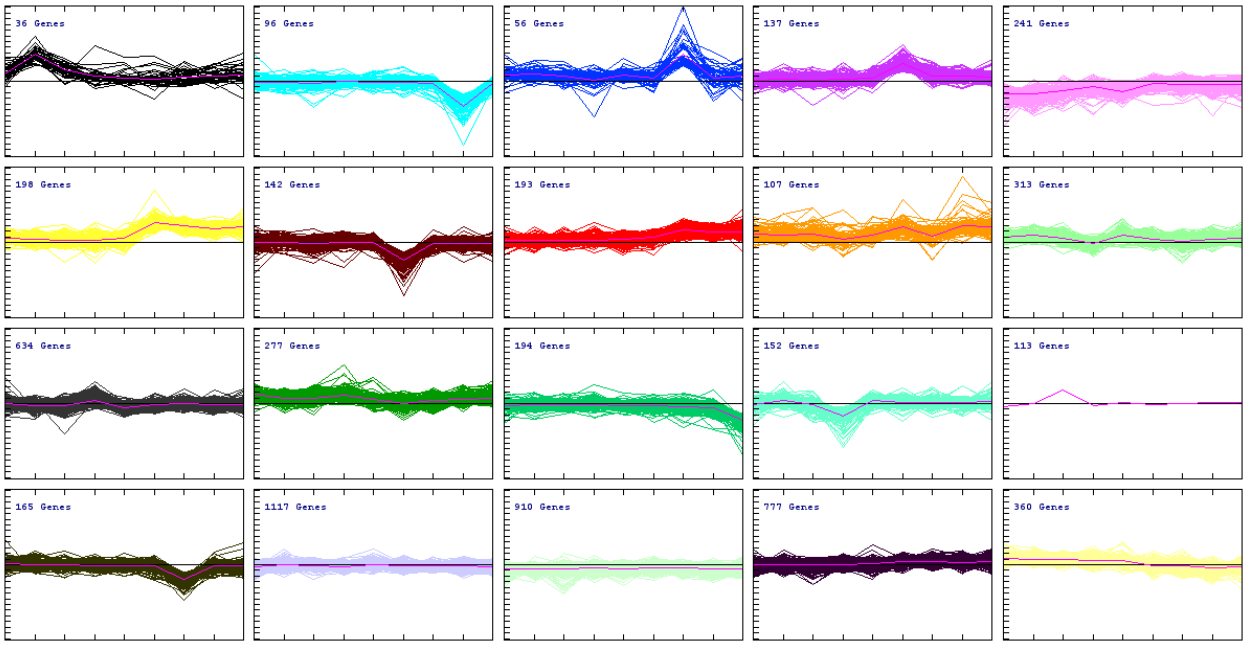


**Fig 3.5.** K-Means/Median clustering of the microarray dataset into 20 clusters. The panels above represent the different clusters and the mean/median expression pattern for each cluster indicated by the pink line. Also indicated are the number of genes in each cluster and their expression profiles.

period and staying at high levels to the end (yellow cluster). The same can also be seen for clusters containing genes undergoing sudden negative effects in transcription at the middle (light blue and brown clusters), as well as the end of fermentation (light blue and dark green clusters). Although not covered by the scope of this article it is possible to try and find co-expressed genes within such clusters and possibly attaching putative functional categories to unknown genes.

Another statistical technique called Pavlides Template Matching or PTM was used to search the dataset for specific expression patterns that could be linked to specific fermentation parameters. In this technique, a specific expression profile is chosen as a template and the dataset is searched for expression vectors that match it. As the first major stress condition that the yeast would encounter during the fermentation is the depletion of free assimilable nitrogen, the first two templates chosen

were those where gene expression is either induced or repressed during the early growth phase (arrays X1 – X3) and remains unchanged during the rest of the fermentation.

In **Figure 3.6** a section of a heat map of the results from the above template matching can be seen. Panel A represent genes induced during the early growth phase and panel B those which are initially repressed. The colours to the right hand side of the heat map represent the clusters obtained with the K-means/Median clustering (**Figure 3.5**). From the data it is clear that the yeast cell is actively increasing its uptake of other sources of nitrogen in the form of amino acids, as transcription of both *VAP1* and *AGP2* – general amino acid permeases, *BAP2* – the high affinity leucine permease and *FUR4* – a uracil permease is up-regulated. Genes involved in protein synthesis are also up-regulated, including: *RPL19B*, *RPL23A*, *RPL32*, *MRP21*, *SUP45*, *PET112*, *CBP6*, and *GRS1*. On the other hand, the following genes, *UBC4*, *UPB14* and *PRE7* involved in protein degradation are also induced. This would indicate that the cells are actively taking up amino acids from the medium and due to the increase in metabolism and growth there is a high turnover of protein synthesis and degradation. Other cellular processes that are represented by several members are cell wall biosynthesis, glucose repression, and transcription. Interestingly some of the genes most repressed during this early growth stage is those involved in amino acid metabolism. These include: *ARG82*, *LYS21*, *GCN4*, *ARO9*, *HIS1*, *CYS4* and *BAS1*. This is an indication of the cellular uptake of amino acids negatively influencing the de novo synthesis of them by the cell. Once again, some genes involved in both protein synthesis and degradation are repressed as well genes involved in cellular processes such as stress response and the metabolism of storage carbohydrates such as glycogen and trehalose. Indeed these results clearly point out the influence that the availability of certain nutrient, in this case nitrogen, has on the transcriptome of the yeast cell.

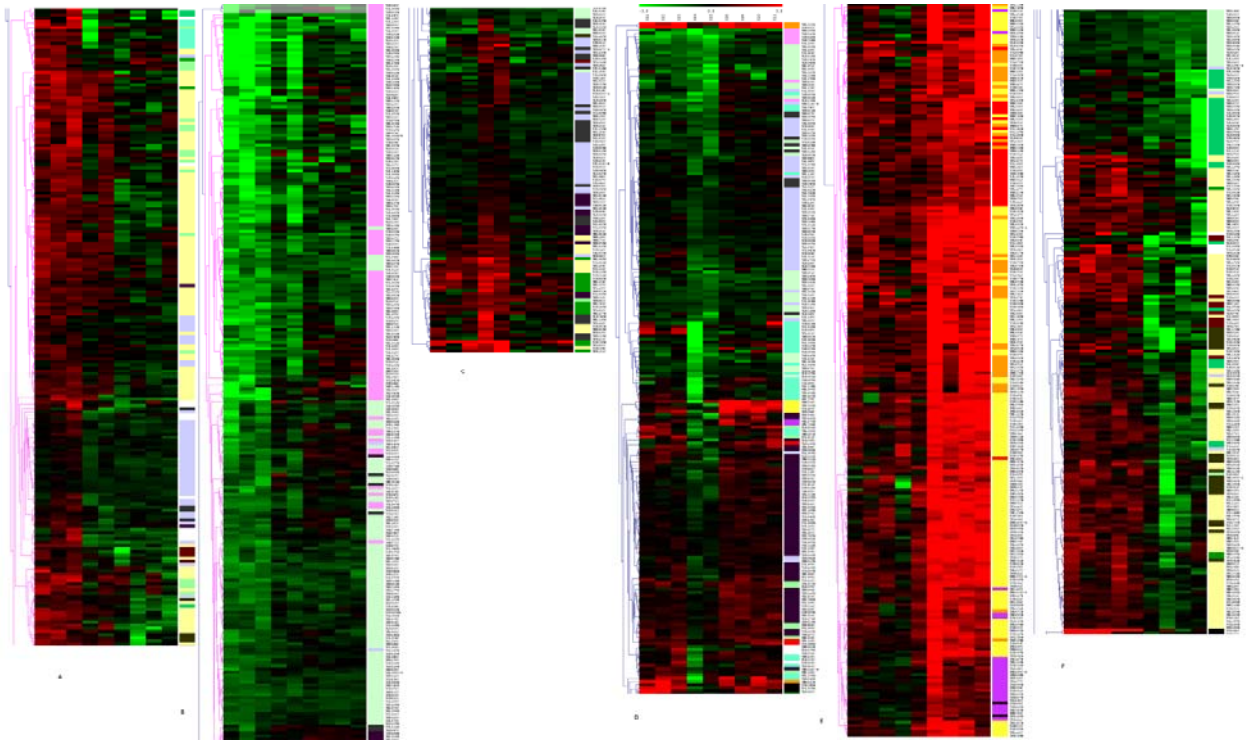

**Fig. 3.6.** Heat maps of sections of the results from PTM analysis of the dataset. Panel A represent genes induced during the early growth phase and panel B those which are initially repressed. Panel C represents genes induced only during the logarithmic growth phase and panel D those which are repressed only during this stage of the fermentation. Panel E represent genes induced during the stationary phase and panel F those genes induced only during the stationary phase. The colours to the right represent the clusters shown in **Figure 3.5**.

PTM was used to search for gene expression profiles that showed either an increase or a decrease in gene expression only during the logarithmic growth phase. In **Figure 3.6** a heat map of a section of these results can be seen. Panel C represent genes induced only during the logarithmic growth phase and panel D those which are repressed only during this stage of the fermentation. The colours to the right hand side of the heat map represent the clusters obtained with the K-means/Median clustering (**Figure 3.5**). Unlike the case for the early growth stages there are no immediate correlations to be drawn regarding fermentation parameters and the transcriptome. However, increased expression of genes such as *CDC19* – a pyruvate kinase, *URA7* – involved in pyrimidine biosynthesis as well as the genes, *YAT1*, *PDR3*, *APL3*, *SEC17*, *PET9* and *FUI1* – all

involved in secretion and transport gives an indication of the high metabolic turnover of the cell during this period. It would seem that conditions are not yet too stressful, as no stress response genes are up-regulated during this period. Interestingly, various genes whose products are involved in key metabolic processes such as glycolysis (*PDC1*, *GLK1*, *PFK21*, *FBP1* and *LAT1*), galactose metabolism (*GAL1*) and the glyoxylate (*ICL1*) and TCA cycle (*FUM1*) are down-regulated during this period. This seems to indicate that although the cell is still very metabolically active during this period, external conditions such as rapidly decreasing levels of glucose and fructose, can decrease expression of genes involved in these pathways, possibly retarding fluxes through them. It is also clear that at this stage the yeast does not perceive its external environment as stressful, as the repression of various stress-related genes such as *SSU81* and *HOG1* (involved in signalling in the high osmolarity pathway) and *YAP1* (involved in oxidative stress), indicate.

In **Figure 3.6** a heat map of the last stages of the model wine fermentation can be seen. Panel E represent genes induced during the late logarithmic and the stationary phase and panel F those genes those genes induced only during the stationary phase. The colours to the right hand side of the heat map represent the clusters obtained with the K-means/Median clustering (**Figure 3.5**). Numerous genes involved in the cell cycle (*CDC15*, *CHK1*, *MBP1*, *SIT4*, *CDC50*, *PPH21* and *PCL2*), DNA repair (*APN2*, *HSM3*, *MGT1*, *RAD59*, *SIR2*, *DUN1* and *MSH3*) as well as various genes involved in protein degradation are induced during this period. This may be due to the gradual decline in metabolic activity of the cells, but increases in the expression of genes involved in cell wall and cytoskeleton structure and maintenance (*SLA1*, *SKT5*, *SRO9*, *ARC40*, *ROT2*, *KCS1*, *ECM21* and *ACT2*) as well as those involved in the biosynthesis of stress protection trehalose (*NTH1* and *TPS2*) is a good indication of the cellular response to ethanol toxicity. Interestingly many genes involved in DNA repair is also down-regulated during this period, including: *HDF2*, *RAD52*, *OGG1*, *PIF1* and *HRR25*. However, there seems to be general downturn in protein synthesis (*RPL37B*, *MRPL37*, *RPS1B*, *RPL6B* and *TIF5*), folding (*NIP1*, *GPI12*, *HSP82*

and *FPR1*), glycosylation (*AMD1* and *MNN9*) and even degradation (*QRI8*, *PUP1* and *PRE5*) implying an imminent cellular senescence followed by death and autolysis (*APG13*).

### 3.4.4 GENE EXPRESSION PROFILING

In **Figure 3.7** a gene expression profile is shown in the form of a heat map for genes previously shown to impact on the Ehrlich pathway – the conversion of branched-chain amino acids to higher alcohols and volatile fatty acids (Styger *et al*., this dissertation Chapters 4 and 5), whilst **Figure 3.8** shows the profile of genes involved in fermentation. It is clear from both **Figures 3.7** and **3.8** that there are no dramatic up- or down-regulated genes amongst the two groups shown. This could indicate that in most cases, genes are induced or repressed by small amounts between closely related time points.

It was previously stated that the loop design of the present experiment would enable one to draw a semi-cumulative gene expression profile for the fermentation period. With semi-cumulative it is meant that a continuous line can be drawn through the gene expression data of the 11 arrays spanning the fermentation period. It is important to stress that this graphical representation in only semi-cumulative, as each array is in fact an individual experiment, with its own experimental errors and the mere fact that each array contains a time point of the previous array is not enough to draw a truly cumulative graph, with absolute gene expression values.

**Figures 3.9** and **3.10** show two such semi-cumulative graphical representations of gene expression profiles, for genes shown to impact on the Ehrlich pathway and fermentation, respectively. It is immediately obvious from **Figure 3.9** that the majority of genes found to impact on the Ehrlich pathway are not significantly induced or repressed during the course of the fermentation. This is consistent with the general trend seen in the discussion of **Tables 3.2** and **3.3** above. However, genes such as *QCR2* and *ADH3* are continuously up-regulated during the fermentation, while *PAD1* shows continuous down-regulation. The reasons for this continuous regulation, either up or

down, are still unclear. This could indicate that the formation of higher alcohols and volatile fatty acids, based on the transcriptomic data, gradually occurs through-out the fermentation and that there are no sudden upwards surges in the levels of these compounds due to sudden transcriptomic shifts. This phenomenon will be discussed in detail in the next section.

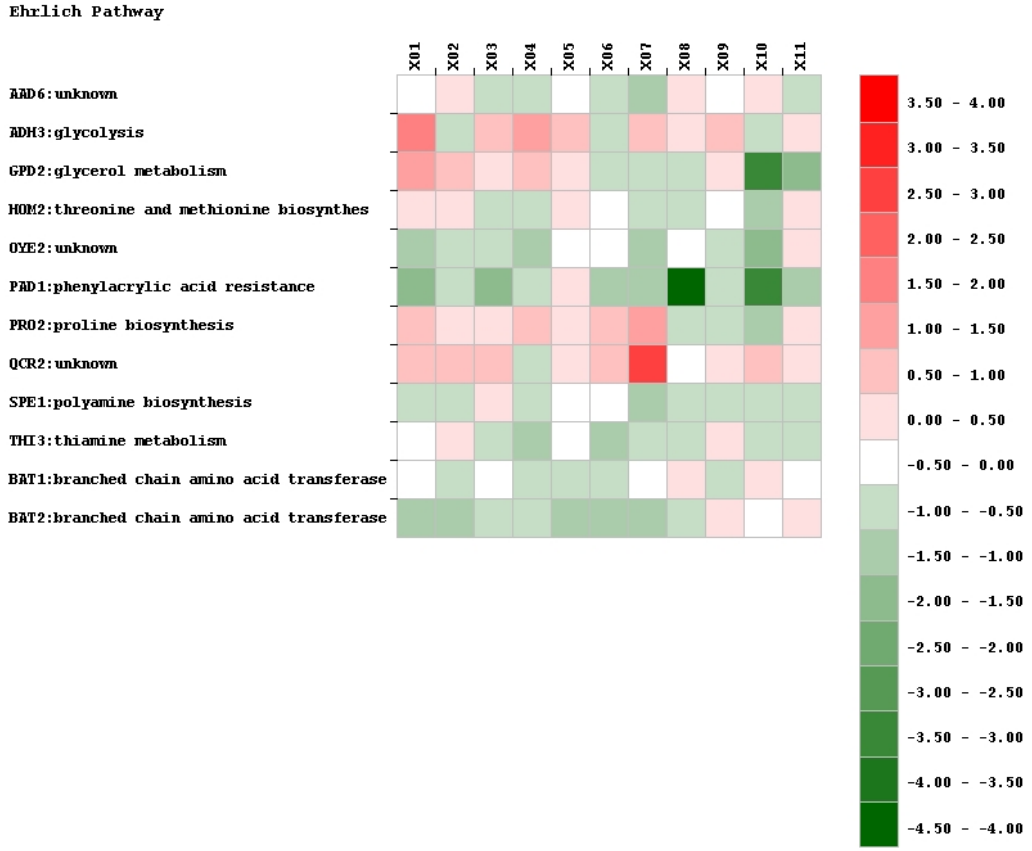


**Fig. 3.7**. Gene expression profile in the form of a heat map for genes previously shown to impact on metabolites of the Ehrlich pathway. The arrays are indicated at the top of the figure and the genes to the left of the figure. The scale to the right indicates the ratio in log2 of the particular time point. Red means an increase in gene expression, whilst green indicates a decrease in gene expression, with the intensity of the colour indicating the size of the increase or decrease.

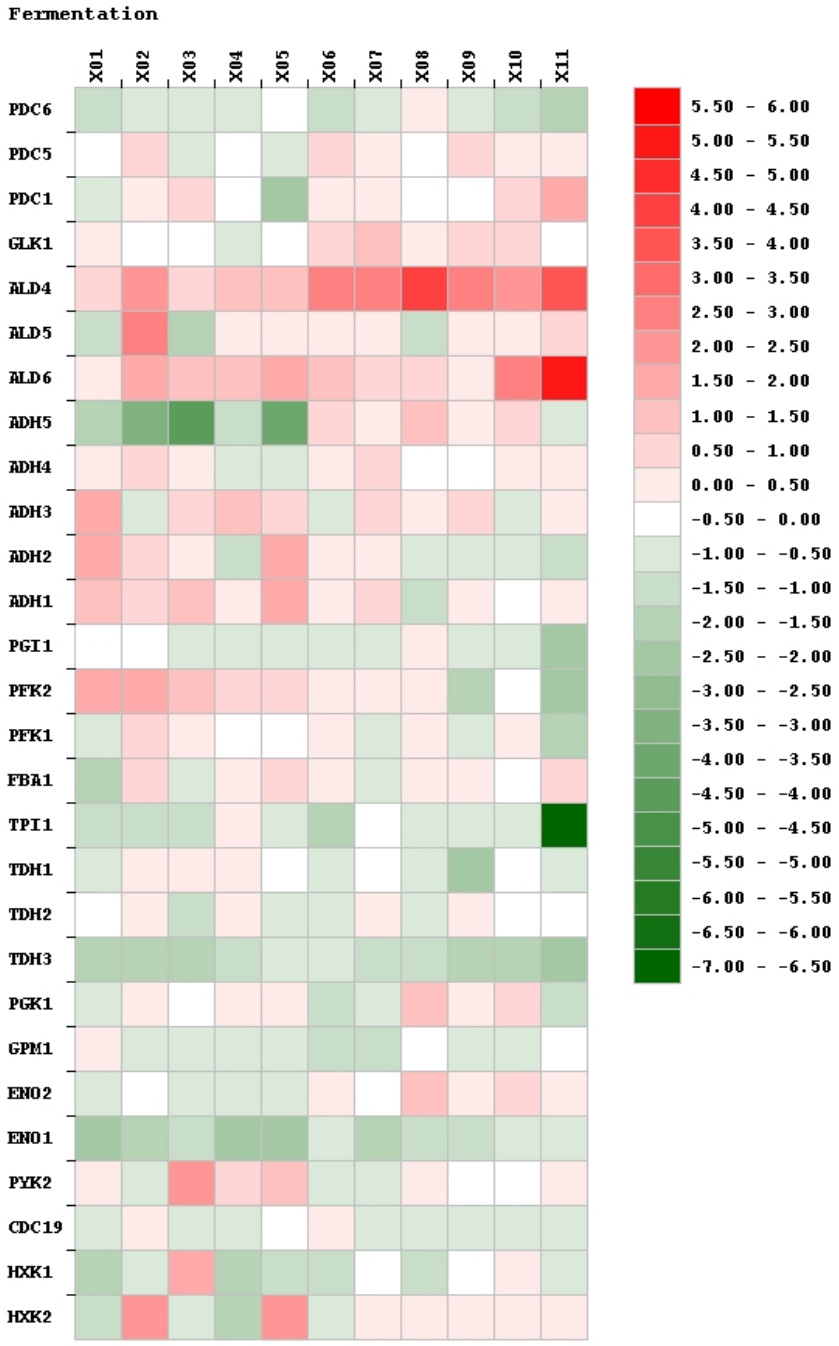


**Fig. 3.8**. Gene expression profile in the form of a heat map for genes involved in the process of fermentation. The arrays are indicated at the top of the figure and the genes to the left of the figure. The scale to the right indicates the ratio in log 2 of the particular time point. Red means an increase in gene expression, whilst green indicates a decrease in gene expression, with the intensity of the colour indicating the size of the increase or decrease.

The genes involved in fermentation, as shown in **Figure 3.10**, show a similar picture. Most of the genes are not significantly up- or down-regulated during the course of the fermentation. Some interesting exceptions are *ALD6*, which expression initially increases before reaching a plateau after about 40 hours. Then at the latter stages of the fermentation (around 120 hours) the gene is suddenly dramatically up-regulated. This gene plays a role in the conversion of acetaldehyde into acetate and it might be up-regulated at the end of fermentation to help remove excess acetaldehyde, or in restoring the redox balance in the cell. A similar gene, *ALD4*, shows continuous up-regulation throughout the fermentation. This only goes to show that there are major differences in the way that certain functionally related genes are regulated in comparison to another group of genes, when compared during the course of a model wine fermentation.

### 3.4.5 INVESTIGATING SUDDEN TEMPORAL CHANGES OR "SPIKES" IN GENE EXPRESSION

Other studies on the transcriptome analysis of wine yeasts used either only six time points during the whole course of the fermentation, used a chemostat to obtain a steady state, or focused on a particular growth stage or fermentation condition. The numerous closely situated time points in this dataset, as well as the experimental loop design – where time points are repeated in different colour channels on subsequent arrays - made it possible to investigate real-time adjustments of the transcriptome. However, it is possible that these changes in gene transcription are rather the effect of a certain threshold being reached and not necessarily due to changes in the environment. The experimental design was chosen not only to cover the entire fermentation time course in high temporal resolution, but enabled the use of advanced statistical analysis of the data to extract biologically relevant phenomena without the need of costly multiple experimental repeats. This study is therefore unique and can be used to elucidate some of the small-scale changes, in terms of time, occurring in the transcriptome of an industrial yeast strain during a model wine fermentation. In order to investigate this aspect, firstly separate normalised time course vectors

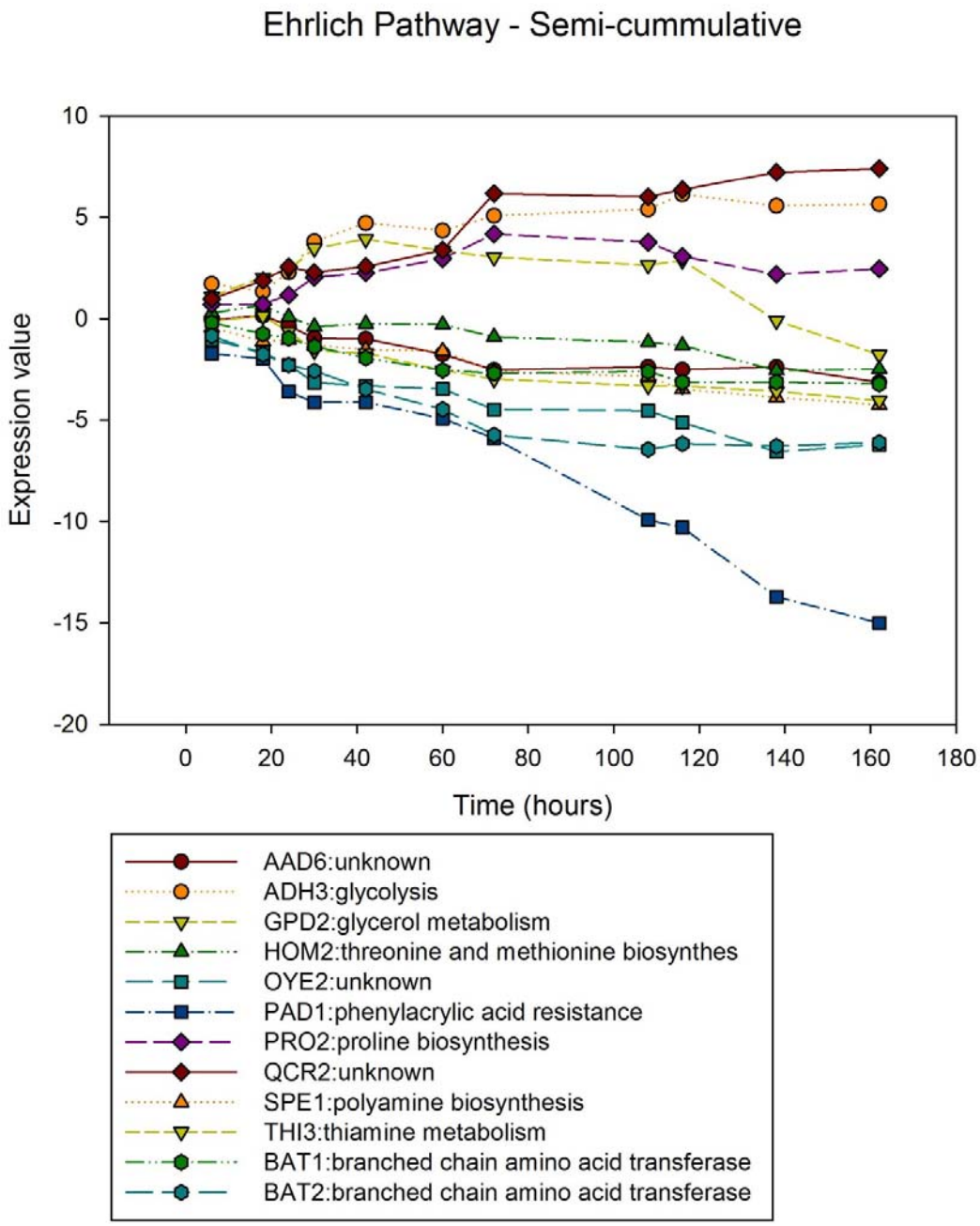


**Fig. 3.9**. Semi-cumulative graphical representation of the gene expression profile of genes thought to be involved in the Ehrlich pathway, during the course of a model wine fermentation. This was generated by summing the concurrent expression values of the genes indicated over the range of arrays, indicating the expression trend of the gene over the course of the model wine fermentation.

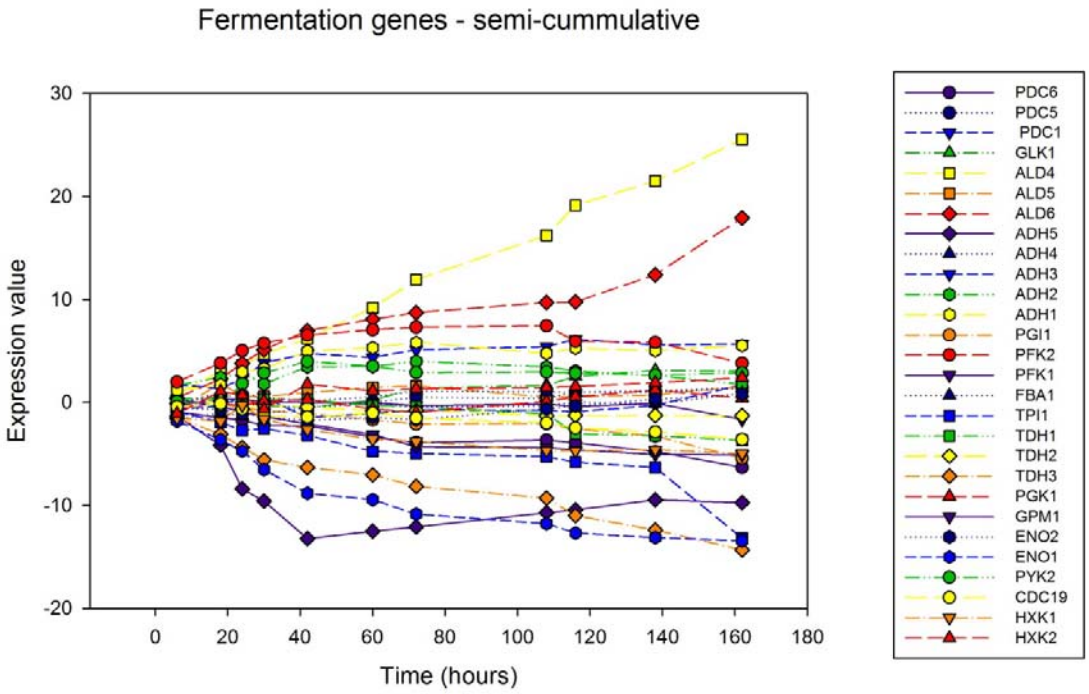


**Fig. 3.10**. Semi-cumulative graphical representation of the gene expression profile of genes involved in the process of fermentation, during the course of a model wine fermentation. This was generated by summing the concurrent expression values of the genes indicated over the range of arrays, indicating the expression trend of the gene over the course of the model wine fermentation.

from both the red and the green channel were constructed and secondly, genes who showed a greater than two fold up- or down-regulation between any two time points and a subsequent greater than two fold inverse change in expression level at the next time point were investigated for both the green and red channels. This would constitute an upward or a downward spike and represent a sudden change in transcriptional regulation in a short space of time. Of the total dataset 1803 genes showed such a response in the red channel and 945 genes in the green channel, with 627 genes common to both channels.

However, this would seem to be an unexpectedly high number of genes up- or down-regulated within such short time periods and might indicate that there are numerous genes undergoing rapid

“spiking” regulatory on and off shifts during the time course of a fermentation that has thus far not been reported due to the lack of high resolution data. Unfortunately given the previously mentioned hybridisation problems with arrays X6 and X7, these “spikes” could also be the result of artefacts underlying the problematic hybridisation and washing quality of the slides. However, by increasing the stringency of the parameters to include only genes that respond in both the green and red channels by at least a Pearson correlation threshold of 0.8, this set was reduced to 401 genes in the red channel and 198 genes in the green channel. This would in some way negate any hybridisation problems. These genes were clustered and in **Figure 3.11** two clusters from both channels are represented. Panels A and B represent clusters from the red channel, whilst panels C and D represent clusters from the green channel.

It is clear from **Figure 3.11** that spikes in expression, in both channels, occur just before (panels B and D) and just after (panels A and C) 20 hours in the fermentation. This period corresponds to the cells entry into logarithmic growth, as well as the almost complete depletion of assimilable nitrogen in the growth medium. When investigating the genes identified with this method it is striking that very few genes relating to any metabolic function are included. Apart from the large number of genes with no known function, the majority of genes encode products involved in transcription, as well as those encoding products playing a role in both DNA and RNA polymerase. Additionally, many genes involved in ribosomal functioning and cell wall and cellular membrane structure also undergo a spike in transcription. Interestingly a large set of genes involved in chromatin structure and remodelling are suddenly up-regulated. This evidence point to the cell rapidly positioning itself to quickly adapt to the changing environment, by reading the machinery needed to quickly transcribe, translate and process any proteins needed to fully take advantage to any environmental condition that might arise. This behaviour has not been reported previously and, if validated with independent real-time PCR analysis, might explain the low levels of correlation normally seen between mRNA and protein levels when comparing experiments of this sort.

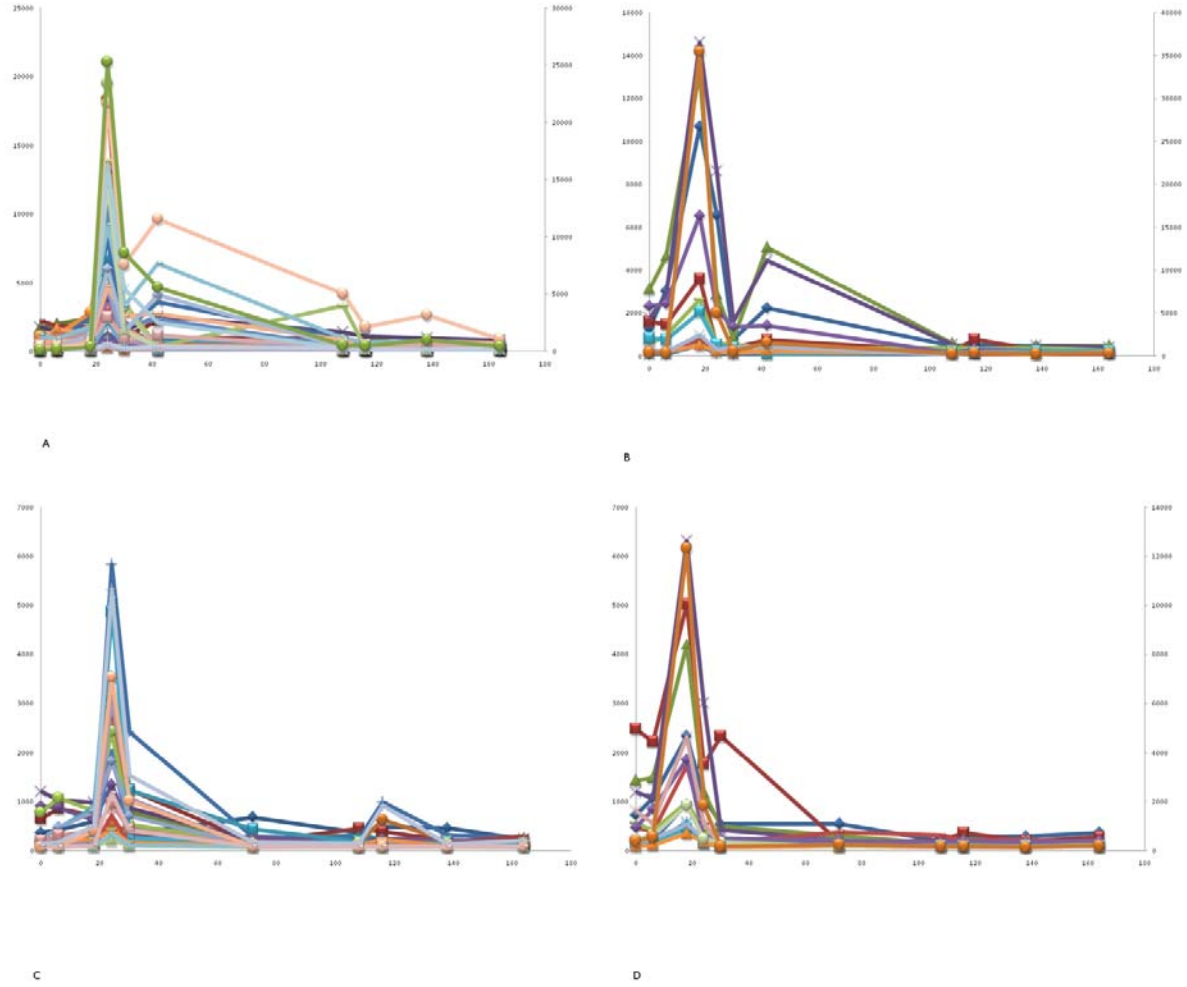


**Fig. 3.11**. Line plots showing clusters of raw expression data from the red channel, panels A and B, as well as the green channel, panels C and D. These genes were chosen because they showed a greater than two fold up- or down-regulation between any two time points and a subsequent greater than two fold inverse change in expression level at the next time point for both the green and red channels. This would constitute an upward or a downward spike and represent a sudden change in transcriptional regulation in a short space of time.

## 3.5 CONCLUSION

The above was a very general introduction into the data generated by a set of microarray experiments conducted on an industrial wine yeast strain, during a model wine fermentation. Altogether 14 arrays were used and a total of 6218 genes were identified from the arrays. Significant changes in the gene expression profile were observed during different growth stages of the yeast cells, during different media composition stages, i.e. in regards to nutrient concentration and stress conditions, and between different functional classes of genes.

Yeast cells undergo a variety of stresses during the course of an alcoholic fermentation. These are normally senses by the cell and various stress responses are enabled. In order to do so the transcription of various genes involved in stress response processes must take place. In this study, the transcriptome of an industrial wine yeast was analysed during the course of a model wine fermentation. In accordance with a previous study by Rossignol *et al*. (2003) [3], we could not find definite evidence of a yeast stress response due to exposure to early stresses such as high osmotic pressure and acidity. However, our results are in correspondence with other researchers when the stress response towards nutrient limitation – specifically nitrogen [12], as well as ethanol toxicity towards the end of the fermentation [14].

The present dataset also shows that the transcriptome of the fermenting yeast is sensitive to fermentation parameters and environmental cues as nitrogen depletion during the early growth phase results in an increase in transcription of genes whose products are involved in the uptake of amino acids into the cell, as well as a down-regulation of genes involved in the de novo synthesis of these compounds. During the logarithmic growth phase no correlation could be found between fermentation parameters and gene expression and it would seem that changes in the transcriptome during this period are largely due to the increased metabolic activity of these cells. However, the presence of high concentrations of ethanol and the depletion of nutrients during the stationary

phase is reflected in the increased transcription of genes involved in cell wall biosynthesis and maintenance as well as the cytoskeleton - perhaps to negate the disruption of the cell wall due to ethanol toxicity. Also induced were genes involved in the metabolism of trehalose, a general cellular stress metabolite that could also play a part in strengthening the cell wall and membrane. It would also seem that the cell readies itself for a period of senescence and ultimately death, by down-regulating genes involved in various protein-related activities.

An even more in depth study of these results - with ongoing statistical evaluations, will ensure that even more valuable biologically significant findings be unearthed. For instance, the metabolite profile, as well as the profile of aromatic compounds, could in future be integrated with the genome analysis in order to investigate whether it is possible to link a certain metabolic event, e.g. the production of an aromatic ester, with an induction or repression of a certain gene or genes.

In conclusion we have generated a highly valuable set of data that will not only benefit the current investigation, but also future investigations.

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# Chapter 4

# Research results

## Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols

**A modified version of this paper is currently being submitted to Applied Microbiology and Biotechnology**

# 4. IDENTIFYING GENES THAT IMPACT ON AROMA PROFILES PRODUCED BY *SACCHAROMYCES CEREVISAE* AND THE PRODUCTION OF HIGHER ALCOHOLS

## 4.1 ABSTRACT

During alcoholic fermentation many volatile aroma compounds are formed by *Saccharomyces cerevisiae*, including esters, fatty acids and higher alcohols. While the metabolic network that leads to the formation of these compounds is reasonably well mapped, surprisingly little is known about specific enzymes involved in specific reactions, the regulation of the network and the physiological roles of individual pathways within the network. Furthermore, different yeast strains tend to produce significantly different aroma profiles. These differences are of tremendous biotechnological interest, since producers of alcoholic beverages such as wine and beer are searching for means to diversify and improve their product range. Various factors that directly or indirectly affect and regulate the network have been identified in the past. Internal factors include the redox, energy and nutritional balance of a cell, which are in turn dependent on and influenced by the external physico-chemical conditions such as precursor availability, nutrient availability, and stress factors such as temperature and osmotic pressure. To gain a better understanding of the regulations and physiological role of this network, we took a functional genomics approach by screening a subset of the EUROSCARF strain deletion library for genes that, when deleted, would impact most significantly on the aroma profile produced under fermentative conditions. The ten genes, whose deletion impacted most significantly on the aroma production network, and more specifically higher alcohol production, were selected and further characterised to assess their mode of action within or on this metabolic network.

## 4.2 INTRODUCTION

The production of aromatic or volatile compounds during alcoholic fermentation by yeast contributes significantly to the quality of products such as wine and beer. The most relevant families of compounds produced by yeast include esters, higher alcohols and fatty acids. These

compounds, and associated metabolites, play an important part in the flavour and aroma of wine [1-3], beer [4, 5], cider [6], as well as that of fermented dairy products such as cheese [7, 8]. Different commercial wine and beer yeast strains produce significantly different aroma compounds, and such strains have in part been selected for their ability to impart specific sensory profiles to the final product [9, 10].

However, the metabolic network responsible for the production of these compounds (**Figure 4.1**) is complex and, while reasonably well mapped, little information about the role of specific genes and their regulation within this network is available. Here we applied a mutant screening approach to identify genes whose products most significantly impact on this network. The primary focus of the work was directed towards identifying genetic factors that affect the conversion of amino acids to aroma compounds. Indeed, many data sets have confirmed that amino acids constitute the most relevant precursors for the biosynthesis of many volatile compounds [11]. The central part of this pathway is also referred to as the Ehrlich reaction [12], and some aspects of the impact of this pathway on aroma production has been studied in the past.

Central carbon and amino acid metabolisms are the major providers of precursor for the production of most relevant impact compounds. During alcoholic fermentation, central carbon metabolism and associated pathways provide $C_2$, $C_3$, $C_4$ and $C_5$ carbon precursors such as pyruvate, oxalacetate and α-ketoglutarate. Amino acids, on the other hand, can be metabolised in several ways. In particular, they can be used as such for protein synthesis, or metabolised into other compounds and used for other purposes and metabolic processes, and serve in particular as providers of amino-groups [13]. When deaminated, the main product of the breakdown of these amino acids is a higher alcohol, or fusel alcohol, so called due to the fact that they have more carbon atoms than ethanol. The process by which amino acids are catabolised into higher alcohols is called the Ehrlich reaction, shown in **Figure 4.1** [14].

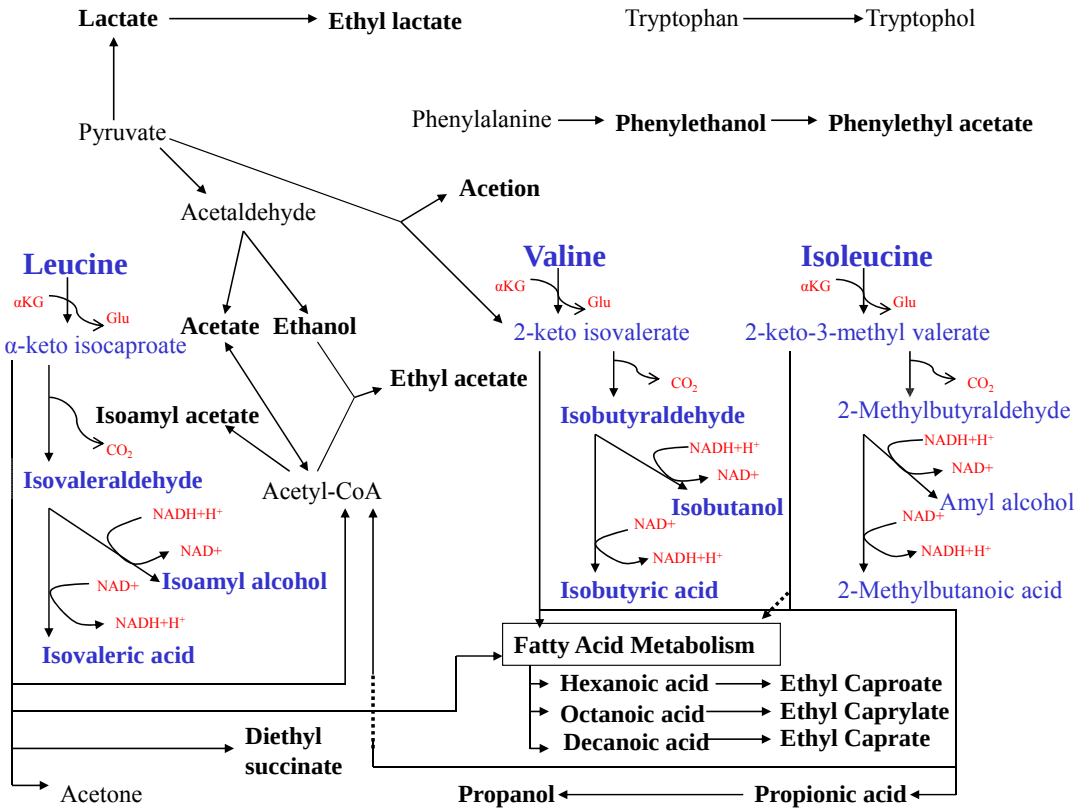


**Fig. 4.1.** A simplified metabolic map of yeast aroma compound production, indicating known metabolic linkages. Bold type indicates aroma compounds important to this study. Compounds shown in blue colour constitute a diagrammatical representation of the Ehrlich pathway, responsible for the production of higher alcohols and volatile acids. Co-factors and transition metabolites are shown in red. αKG is α-keto glutamate and Glu is glutamate.

The branched-chain amino acids, leucine, isoleucine and valine for example, are transported into the yeast cell via several transport systems [15, 16]. Under conditions of nitrogen limitation, these amino acids are used to support the synthesis of other nitrogen-containing metabolites. This transamination reaction is catalysed by mitochondrial and cytosolic branched-chain amino acid transferases (BCAATases) encoded by the *BAT1* and *BAT2* genes [17-22]. In this manner α-keto isocaproic acid is formed from leucine, α-keto isovaleric acid from valine, and α-keto-β-methyl valeric acid from isoleucine [23-26]. Yeast, however, can also generate these α-keto acids through the so-called anabolic pathway, from glucose via pyruvate [23, 24, 27]. However, Chen *et al.* [27] has shown that during a model beer fermentation, the Ehrlich pathway contributed 75% and 85%, respectively to the formation of their corresponding higher alcohols in the case of isoleucine and leucine.

The following step in the Ehrlich pathway is the decarboxylation of the α-keto acid into an aldehyde [28]. Thus, isovaleraldehyde is formed from α-keto isocaproic acid, isobutyraldehyde from α-keto isovaleric acid and 2-methyl butyraldehyde is formed from α-keto-β-methyl valeric acid [23-25, 29]. It has been suggested that the pyruvate decarboxylase genes - *PDC1*, *PDC5* and *PDC6*, may play a part in this decarboxylation reaction, but they are not essential [30, 31]. Other possible decarboxylase genes that could be involved in the decarboxylation of these α-keto acids are *KID1/THI3* and *ARO10* [32-34].

The Ehrlich pathway now splits in two and the final fate of the branched-chain amino acid is thought to depend on the redox status of the yeast cell [33]. The aldehyde can either be reduced via a NADH-dependent reaction to its respective higher alcohol, i.e. isoamyl alcohol is formed from isovaleraldehyde, isobutanol is formed from isobutyraldehyde and active amyl alcohol is formed from 2-methyl butyraldehyde [23-25]; or it can be oxidized via a $NAD^+$ dependent reaction into a volatile acid. If this occurs, iso-valeric acid is formed from isovaleraldehyde, iso-butyric acid is formed from isobutyraldehyde and 2-methyl butanoic acid is formed from 2-methyl butyraldehyde [23-25]. It has been suggested that an alcohol dehydrogenase may catalyze this reductive reaction and an aldehyde dehydrogenase the oxidation reaction [32, 34].

The redox duality of these last two steps of the Ehrlich pathway has given rise to the hypothesis that higher alcohols or volatile acids are formed to help maintain the $NADH/NAD^+$ ratio and so the redox balance of the cell [35, 36]. Some authors, however, believe that the cell produces enough other redox equivalents such as ethanol, glycerol, acetate, acetaldehyde and succinate to fulfil this role [37]. Other hypothesis for higher alcohol production is the removal of toxic aldehyde compounds or as an alternative source for the cell to obtain nitrogen [37, 38].

Previous work showed that the gene products of *BAT1* and *BAT2* were responsible for the first step in the pathway and that deletion of these genes had an impact on the formation of higher

alcohols [22]. Changes in the concentrations of other, seemingly unrelated, aroma compounds were also observed, highlighting the complexities of the interconnections within such complex metabolic networks [22]. In order to identify genes that have the most important impact on the metabolic flux through this network, the yeast deletion library was used to screen for genes that might be involved in the different biochemical reactions of the pathway, i.e. decarboxylases, dehydrogenases and reductases (**Table 4.1**). Genes whose deletion caused the strongest decrease in the production of higher alcohols or related metabolites were further investigated. Thus, 10 genes were obtained whose products seem to be either directly or indirectly involved with the Ehrlich pathway. Further in-depth study of these genes in medium more resembling wine and under a more stringent statistical experimental set-up was performed and results show how they impact on the formation of higher alcohols and related compounds.

## 4.3 MATERIALS AND METHODS

### 4.3.1 STRAINS AND GROWTH CONDITIONS

All strains used where obtained from the EUROSCARF deletion library, except the *BAT1* and *BAT2* over-expression (*pBAT1* and *pBAT2*) and deletion strains (*dbat1* and *dbat2*) that were previously generated [22]. The generation of deletions in the newly identified genes is described below. The media used through-out this study was synthetic complete dextrose (SCD) media [39], with the following changes or additions: SCD+++ medium contained 2 % (w/v) glucose, standard concentrations of all amino acids, except increased concentrations (150 mg/l) of each of the branched-chain amino acids, leucine, isoleucine and valine. SCD5 medium contained standard concentrations of all amino acid, but 5 % (w/v) glucose, whereas SCD5+++ medium had the same increased concentrations of the branched-chain amino acids as SCD+++ medium, but with a glucose content of 5 % (w/v). Initial screening experiments were done in duplicate, whilst follow-up experiments were performed in triplicate.

Strains were grown overnight in 5 ml of YPD medium and inoculated into the sample culture at an optical density at 600 nm of 0.1. Sample cultures consisted of 80 ml medium in 200 ml glass bottles and were placed on a shaking incubator at 30 ºC. The bottles were sealed with only tin-foil and no fermentation caps, resulting in self-anaerobic growth. Weight loss was measured each day to determine the rate and stage of fermentation. After 7 days, a final OD was taken to ensure that results could be normalized, and the cell suspension was centrifuged at 5000 rpm for 5 min. The cell-free supernatant was used for gas chromatographic analysis.

**Table 4.1**. Genes involved in the primary screen for possible involvement in the Ehrlich pathway. Genes marked with (*) indicate those selected after the screening

| **Dehydrogenases** | | | **Decarboxylases** | **Reductases** | |
|---|---|---|---|---|---|
| *AAD3* | *GPD1* | *MTD1* | *GAD1* | *AHP1* | *MET16* |
| *AAD4* | *GPD2** | *OYE2** | *GCV1* | *AYR1* | *MXR1* |
| *AAD6** | *GUT2* | *OYE3* | *GCV2* | *CBR1* | *OAR1* |
| *ADH1* | *HIS4* | *PDA1* | *PAD1** | *COR1* | *PRO2** |
| *ADH2* | *HOM2** | *PDB1* | *PDC1* | *ECM17* | *QCR2** |
| *ADH3** | *HOM6* | *PDX1* | *PDC5* | *ERG24* | *QCR8* |
| *ADH4* | *IDH1* | *PUT1* | *PDC6* | *ERG4* | *RNR1* |
| *ADH5* | *IDH2* | *PUT2* | *PSD2* | *ETR1* | *RNR3* |
| *ADH6* | *IDP1* | *SDH1* | *SPE1** | *FRE1* | *SPS19* |
| *ADH7* | *IDP2* | *SDH2* | *THI3** | *FRE2* | *SRX1* |
| *ALD2* | *IDP3* | *SDH4* | | *FRE3* | *TRR2* |
| *ALD3* | *IMD3* | *SER3* | | *GRE3* | |
| *ALD4* | *IMD4* | *SER33* | | *GRX2* | |
| *ALD5* | *KGD1* | *SFA1* | | *HMG1* | |
| *ALD6* | *KGD2* | *TDH1* | | *HMG2* | |
| *ARA1* | *LAT1* | *TDH2* | | *LYS2* | |
| *DLD3* | *LPD1* | *TDH3* | | *LYS5* | |
| *GDH2* | *LYS12* | *TYR1* | | *MCR1* | |
| *GDH3* | *MDH1* | *URA1* | | *MET10* | |
| *GND1* | *MDH2* | *XYL2* | | *MET12* | |
| *GND2* | *MDH3* | *ZWF1* | | *MET13* | |

### 4.3.2 RECOMBINANT DNA AND PLASMID CONSTRUCTION

Standard procedures for isolation and manipulation of DNA were used throughout this study [40]. Restriction enzymes, T4 DNA ligase (Roche) and ExTaq DNA polymerase (Fermentas) were used in the enzymatic manipulation of DNA according to the specifications of the supplier.

Deletion strains were generated by using the set of primers listed in **Table 4.2**. The yeast disruption plasmid, YDp-U, containing the *URA*3 gene was used as template for PCR with primers designed to amplify the *URA*3 gene, with 50 base pair (bp) overhangs, specific to either the 5' or 3' flanking sequences of the ten genes, i.e. *AAD6, ADH3*, *GPD2*, *HOM2*, *OYE2*, *PAD1, PRO2*, *QCR2, SPE1* and *THI3*. Resulting disruption cassettes were transformed into BY4742 yeast cells, and successful transformants would insert a functional *URA*3 gene into the target gene. Transformants were selected on SC solid medium (2% agar, Difco) without uracil.

**Table 4.2:** Primers used in this study

| Primer name | Sequence | Enzyme |
|---|---|---|
| YDp-AAD6-F | 5'-ATGGCTGATTTATTTGCTCCTGCTCCTGAACCATCTACAGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-AAD6-R | 5'-TTCAACAGGTTCCATTTACCTTGATAGATGCTAAAAGGGGGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-ADH3-F | 5'-ATGTTGAGAACGTCAACATTGTTCACCAGGCGTGTCCAACGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-ADH3-R | 5'-TATTATTTACTAGTATCGACGACGTATCTACCCAAAATCTGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-GPD2-F | 5'-ATGCTTGCTGTCAGAAGATTAACAAGATACACATTCCTTAGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-GPD2-R | 5'-TATTCGTCATCGATGTCTAGCTCTTCAATCATCTCCGGTAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-HOM2-F | 5'-ATGGCTGGAAAGAAAATTGCTGGTGTTTTGGGTGCTACTGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-HOM2-R | 5'-CCTTAAATCAAGTTTCTTGCTAGTAAGATTTCGGCAATCAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-OYE2-F | 5'-ATGCCATTTGTTAAGGACTTTAAGCCACAAGCTTTGGGTGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-OYE2-R | 5'-ATTTTTGTCCCAACCGAGTTTTGAGCTTCTTCGTACGTAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-PAD1-F | 5'-ATGCTCCTATTTCCAAGAAGAACTAATATAGCCTTTTTCAGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-PAD1-R | 5'-GTTACTTGCTTTTTATTCCTTCCCAACGAGGAAAAGTGTCGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-PRO2-F | 5'-ATGTCCAGTTCACAACAAATAGCCAAAAATGCCCGTAAAGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-PRO2-R | 5'-TATAATGTCACAGTCTTTATATCTAAATCCTTGTGAACAAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-QCR2-F | 5'-ATGTTGTCAGCAGCTAGATTGCAATTTGCCCAGGGGTCAGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-QCR2-R | 5'-CTCTTACAATTCGTCCAAATATGGCAAGTTGGAAACATCAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-SPE1-F | 5'-ATGTCTAGTACTCAAGTAGGAAATGCTCTATCTAGTTCCAGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-SPE1-R | 5'-CAATCGAGTTCAGAGTCTATGTATACTATATCCGCAGTCTGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-THI3-F | 5'-ATGAATTCTAGCTATACACAGAGATATGCACTGCCGAAGTGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-THI3-R | 5'-TCAGTATCCAACTTGATTTTTTTTTAGAAGTGGTTGGAATGCTGCAGGTCGACGGATCCG-3' | - |

* The region homologous to the corresponding genes are underlined

### 4.3.3 GAS-CHROMATOGRAPHIC ANALYSIS

Extraction of volatile compounds from the cell-free supernatant was done by liquid-liquid extraction. To each sample of 5 ml cell-free supernatant, 0.1 µl of an internal standard (4-

methyl-2-pentanol, 500 mg/l in 12% (v/v) ethanol) was added. The mixture was extracted with 1 ml of diethyl ether for 5 min in an ultrasonic bath, followed by centrifugation at 4000 rpm for 3 min. The diethyl ether layer was removed and dried on anhydrous sodium sulphate ($Na_2SO_4$) before being injected into the gas chromatograph.

The analysis of volatile compounds was carried out on a Hewlett Packard 6890 gas chromatograph equipped with a split-splitless injector and a flame ionisation detector (FID). A DB-FFAP capillary GC column (Agilent Technologies, Little Falls, Wilmington, USA) with dimensions 60 m length $\times$ 0.25 mm internal diameter with a 0.5 μm film thickness, was used for separation. Hydrogen was used as the carrier gas at a constant flow rate of 3.3 ml/min. The injector temperature was 200 °C, the split ratio 15:1, the split flow rate 49.5 ml/min, and injection volume of 3 μl. The FID was operated at 250 ºC. The oven temperature program was as follows: 33°C (17 min) to 240°C (5 min) at 12°C/min. For each of the compounds analyzed, an internal calibration curve was constructed using known amounts of authentic standards. The internal standard and the chemicals were sourced from Merck (Cape Town, South Africa).

### 4.3.4 INVESTIGATION OF METABOLIC INTERACTIONS

In order to investigate the metabolic effects of gene deletions, a metabolic map of the formation of these compounds was created. The software program GenMapp [41, 42] was used to modify and adapt the already existing map of isoleucine, leucine and valine degradation from the Kyoto Encyclopaedia of Genes and Genomes (KEGG). Although the main function of this program is to overlay microarray data onto metabolic pathways, it is also possible to design novel, or specialist gene databases. The data obtained from the GC analysis of the different deletion strains were treated as a pseudo-microarray. The ratio of deletion strain sample to the wild type sample was calculated and transformed to log2 in order to represent up- and downward trends equally. Microarray visualisation software from The Institute for Genomic Research (TIGR), TMev, was used to visualise the GC data and to cluster deletion strains with regards to their metabolic profile. A gene database where the aroma compounds were treated as genes was

compiled and linked to the metabolic map. Lastly the transformed GC data was fitted onto the map and any possible causal interactions were investigated.

### 4.3.5 STATISTICAL ANALYSIS

The statistical differences between the deletion strains and the wild type strains on the GC data were determined using the Student t-test where the $\rho$-value is determined, and where a $\rho$ value of $\leq 0.05$ would mean that the difference was significant. Multivariate data analysis of the dataset in the form of principle component analysis (PCA) was performed with the Unscrambler software suite.

## 4.4 RESULTS

### 4.4.1 SCREENING OF SELECTED DELETION MUTANTS

Previous work [22] involving the deletion and over-expression of *BAT* genes showed immediate and clear effects on concentrations of higher alcohols and related compounds, as well as the overall aroma profiles, formed during small scale batch fermentations in synthetic medium. In order to discover genes that had the same effect on the aroma profile of the fermented product and which might be involved in or impact on the Ehrlich pathway, it was decided to use the yeast deletion library to screen for mutants that have a significant effect on branched-chain amino acid catabolism.

Due to the chemical nature of the Ehrlich reaction, it was decided to focus the attention of the screening procedure on genes involved in biochemical reactions that are part of this pathway. Thus the Saccharomyces Genome Database (www.yeastgenome.org) was used to identify genes that are involved in similar biochemical reactions, i.e. decarboxylase, dehydrogenase and reductase reactions. **Table 4.1** lists the deletion strains that were selected for the primary screening procedure. The total number of deletion strains screened was 104, including 10

strains deleted for decarboxylase genes, 64 strains deleted for dehydrogenase genes and 33 strains deleted for reductase genes. Consensus protein motifs were established from confirmed proteins relating to decarboxylase, dehydrogenase and reductase catalytic sites. These motifs, as well as gene sequence homology was used to search the *Saccharomyces cerevisiae* genome database for possible candidate genes to include in the screening process. Several putative genes and genes with unknown function were thus included in the screen, selected on this basis of, but none produced aroma profiles different from the wild type strain (data not shown).

In order to visually highlight differences between strains in the large dataset, the Gas Chromatographic (GC) data was treated as a pseudo-microarray and visualized as a heat map. This was done by calculating the ratio of the measured aroma compounds of the deletion strains and of the wild type strain. This ratio was then normalised by log2 to ensure that all fold differences were of an equal value, with positive values indicating a higher concentration of a certain metabolite in the deletion strain compared to the wild type, whilst a negative value would mean that the metabolite was more abundant in the wild type strain than in the strain with the gene deletion.

**Figure 4.2** shows the result of hierarchical clustering, and reveals the patterns in the relationships between metabolite production and gene deletion. It is clear that ethyl acetate, isoamyl alcohol, isobutanol, iso-butyric acid, 2-phenyl ethanol and propanol formed a cluster, while acetic acid, iso-valeric acid and propionic acid formed a second cluster. In the first cluster, only isoamyl alcohol, isobutanol, iso-butyric and propanol are compounds that are directly linked to branched-chain amino acid metabolism and iso-valeric acid and propionic acid in the second cluster. The significance of these clusters is unclear, but hints at the underlying interconnectedness of the aroma production network in **Figure 4.1**, as previously shown [22].

Apart from the expected effects on the production of higher alcohols and related compounds, the screening of deletion strains produced varied results regarding the production of other

aroma- and related compounds as shown in **Figure 4.3**. Production of phenyl ethanol was not affected as dramatically and most strains produced amounts comparable to the wild type. However, acetic acid production varied mostly in the dehydrogenase functional category – likely due to redox effects resulting from gene deletions. Ethyl acetate is an important aroma ester and significantly lower levels were produced by most deletion strains across the functional categories, with the exception of one decarboxylase (*GCV2*) and one reductase (*ECM17*) strain. For the far downstream products of branched-chain amino acid catabolism, propionic acid and propanol, a large spread of production levels was seen – from zero in some instances to values in the range of 20 – 50 mg/L. These results highlight the fact that selection for one or two criteria, would not automatically result in a uniform distribution of attributes with regards to other compounds, and again highlights the complexity of the metabolic network.

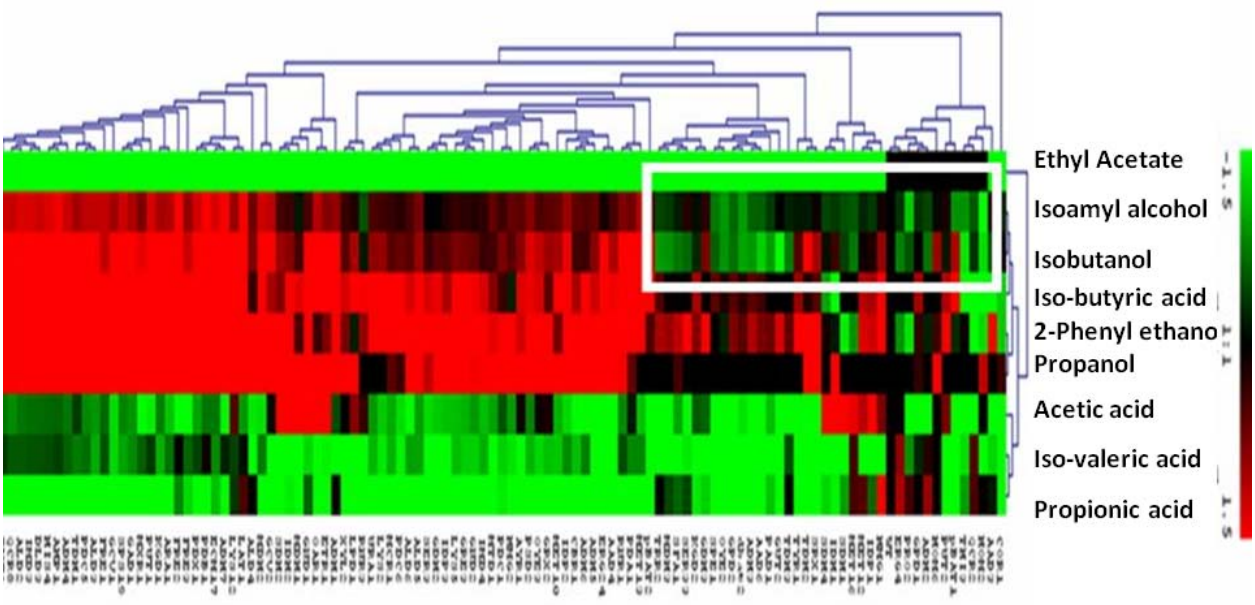


**Fig. 4.2.** A colour-coded diagrammatic representation of the Gas Chromatographic (GC) dataset as a pseudo-microarray, with hierarchical clustering of both the deletion strains and the metabolic compounds produced. The scale on the right hand side displays the ratio of the deletion strain to that of the wild type strain in log2 format. This would mean that a bright green square in the diagram has a ratio of -1.5, meaning that the value for this particular metabolite (indicated in the right hand side of the figure) is in fact 2.8 fold less than found in the wild type strain. The reverse is thus also true for the red squares. The gene names are indicated at the bottom of the figure, but due to the large number of genes in the dataset and the global overview of this figure, it was not possible to make them any clearer.

In order to focus the analysis, the following criteria were used to select genes that appear to most strongly impact on the Ehrlich pathway: The deletion had to lead to large decreases in the production of higher alcohols such as isoamyl alcohol and isobutanol, and be associated with decreases in either iso-valeric acid or iso-butyric acid. The genes that best matched these criteria were further evaluated and are highlighted as the area marked with a white square in **Figure 4.2**. The GC data for the major volatile metabolites produced by these deletion strains in the standard growth conditions are represented as a heat-map in **Figure 4.4**. The results represent the average of duplicate values, but no statistical analysis was performed on the data. The suggested metabolic functions and locations of the ten candidate genes are shown in **Table 4.3**. Of the ten genes selected, five are classified as dehydrogenases, three as decarboxylases and two as reductases. The dehydrogenase genes include genes involved in major cellular redox maintenance reactions, such as *GPD2* and *ADH3*, as well as general redox reactions in the case of *OYE2*. This is an indication of the importance of redox homeostasis on the formation of these compounds. The remaining dehydrogenases, *AAD6* and *HOM2*, do not play a significant role in this regards and are possibly directly involved in the Ehrlich reaction. The three decarboxylases *PAD1*, *SPE1* and *THI3*, catalyse reactions with substrates similar to those of the Ehrlich pathway and might exert their function directly on the pathway, or as promiscuous enzymes. The same could also apply for the two reductase genes, *PRO2* and *QCR2*.

**Table 4.3.** List of the ten candidate genes chosen after the initial screen and their attributes

| Gene | ORF | Function | Biological Process | Cellular Component |
|---|---|---|---|---|
| *AAD6* | YFL056C | Aryl-alcohol dehydrogenase (putative) | Aldehyde metabolism | Unknown |
| *ADH3* | YMR083W | Alcohol dehydrogenase isoenzyme III | NADH oxidation, fermentation | Mitochondrion |
| *GPD2* | YOL059W | Glycerol-3-phosphate dehydrogenase | NADH oxidation, glycerol metabolism | Cytosol, Mitochondrion |
| *HOM2* | YDR158W | Aspartic β semi-aldehyde dehydrogenase | Homoserine biosynthesis, methionine & threonine metabolism | Cytoplasm, Nucleus |
| *OYE2* | YHR179W | NAPDH dehydrogenase (old yellow enzyme), isoform II | Unknown | Cytoplasm, Nucleus, Mitochondrion |
| *PAD1* | YDR538W | Phenylacrylic acid decarboxylase | Aromatic compound metabolism | Mitochondrion |
| *PRO2* | YOR323C | Γ-Glutamyl phosphate reductase | Proline biosynthesis | Cytoplasm, Nucleus |
| *QCR2* | YPR191W | 40 kDa ubiquinol cytochrome-c reductase core protein II | Aerobic respiration, mitochondrial electron transport | Mitochondrion |
| *SPE1* | YKL184W | Ornithine decarboxylase | Pantothenate, putrescine biosynthesis | Cytoplasm |
| *THI3* | YDL080C | α-ketoisocaproate decarboxylase | Thiamin biosynthesis | Nucleus |

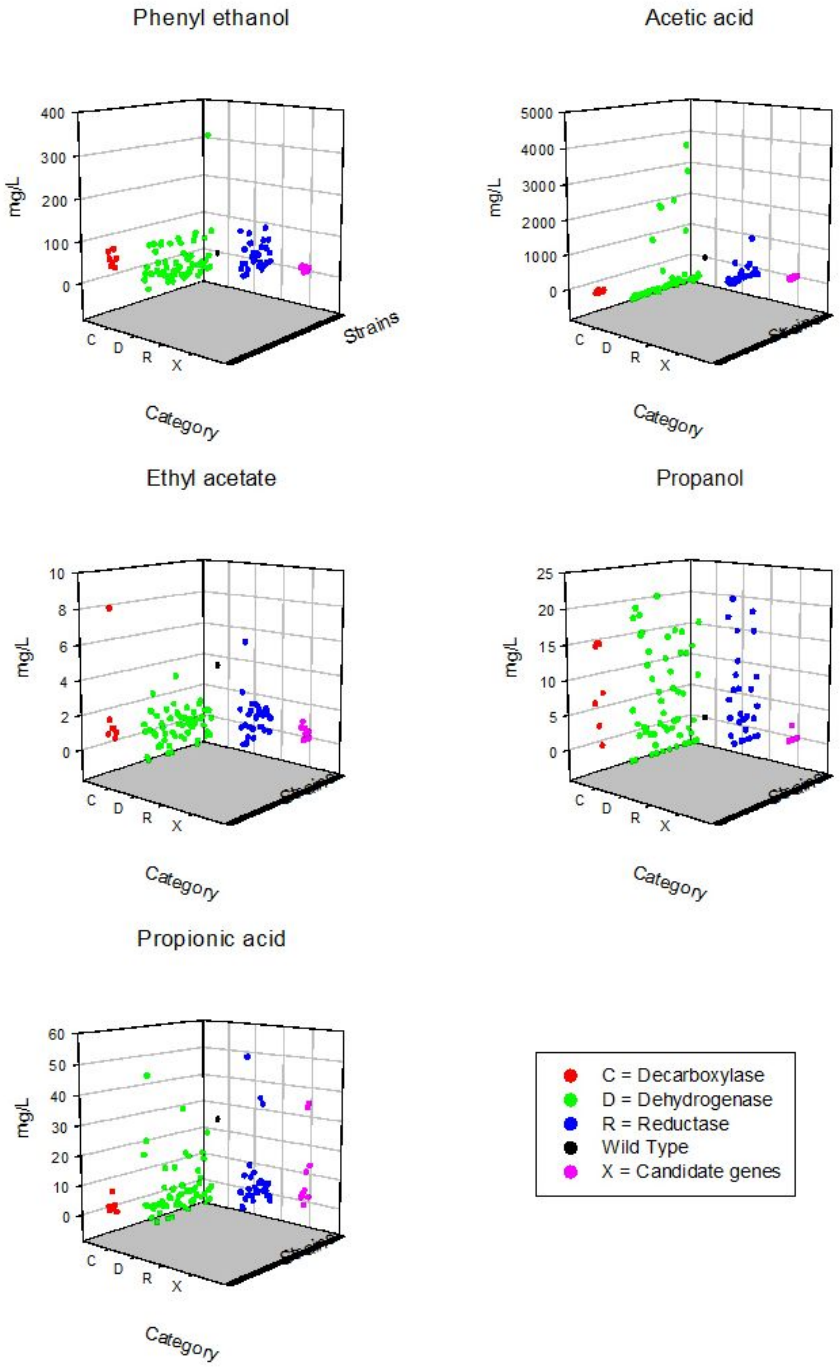


**Fig. 4.3.** 3D scatter plot of the GC data from the entire deletion library screening dataset, indicating the end levels of various chemical compounds not directly related to branched-chain amino acid metabolism. The strains are not indicated on the graphs, due to lack of space, but are divided into their functional categories, i.e. red dots represent decarboxylase (C) genes, green dots represent dehydrogenase (D) genes and blue dots represent reductase (R) genes. The wild type levels are indicated by a black dot and the purple (X) dots represent the ten genes chosen to study further.

**Figure 4.4** clearly indicates that all ten genes show a significant impact on the majority of metabolites measured. In most strains, acetic acid and ethyl acetate production were significantly reduced, suggesting that the gene products may impact on the redox balancing potential of the corresponding strains. All selected strains also showed a decrease in the level of isoamyl alcohol and isobutanol produced when compared to the wild type strain, with the *HOM2* deleted strain showing a fourfold decrease. Indeed the levels of most of the deletion strains chosen are near to that of the *BAT2* deletion strain, *dbat2*, that was previously shown to have a significant effect on the production of higher alcohols and their related metabolites [22]. In contrast the *BAT2* over-expression strain, *pBAT2*, shows an increase in the concentrations of these compounds, with little effect seen for the *BAT1* over-expression strain, *pBAT1*, as well as the *BAT1* deletion strain, *dbat1*. This is in line with previous data [22].

The effects on the production of the volatile branched chain acids are less clear-cut, especially in the case of iso-butyric acid, where only three deletion strains, Δ*hom2*, Δ*qcr2* and Δ*thi3* show pronounced effects. However, all deletion strains except Δ*qcr2* show a decrease in the amount of iso-valeric acid production, hinting at a possible specificity of certain gene products to certain metabolites in the Ehrlich pathway.

### 4.4.2 THE EFFECT OF GENE DELETION ON THE PRODUCTION OF METABOLITES INVOLVED IN THE EHRLICH PATHWAY

The initial previous screening experiment described above was performed using deletion strains from the EUROSCARF library, and it has been shown that it is not always possible to replicate results from EUROSCARF strains when using strains where the gene(s) had been knocked-out using other deletion cassettes [43]. It was thus important to independently delete the 10 candidate genes and re-asses their impact on the metabolism of higher alcohols and related compounds. Indeed, several reports and findings in our own laboratory suggest that a significant number of phenotypes of EUROSCARF deletion strains may not be directly due to the deleted gene, but be due to secondary mutations in those strains.

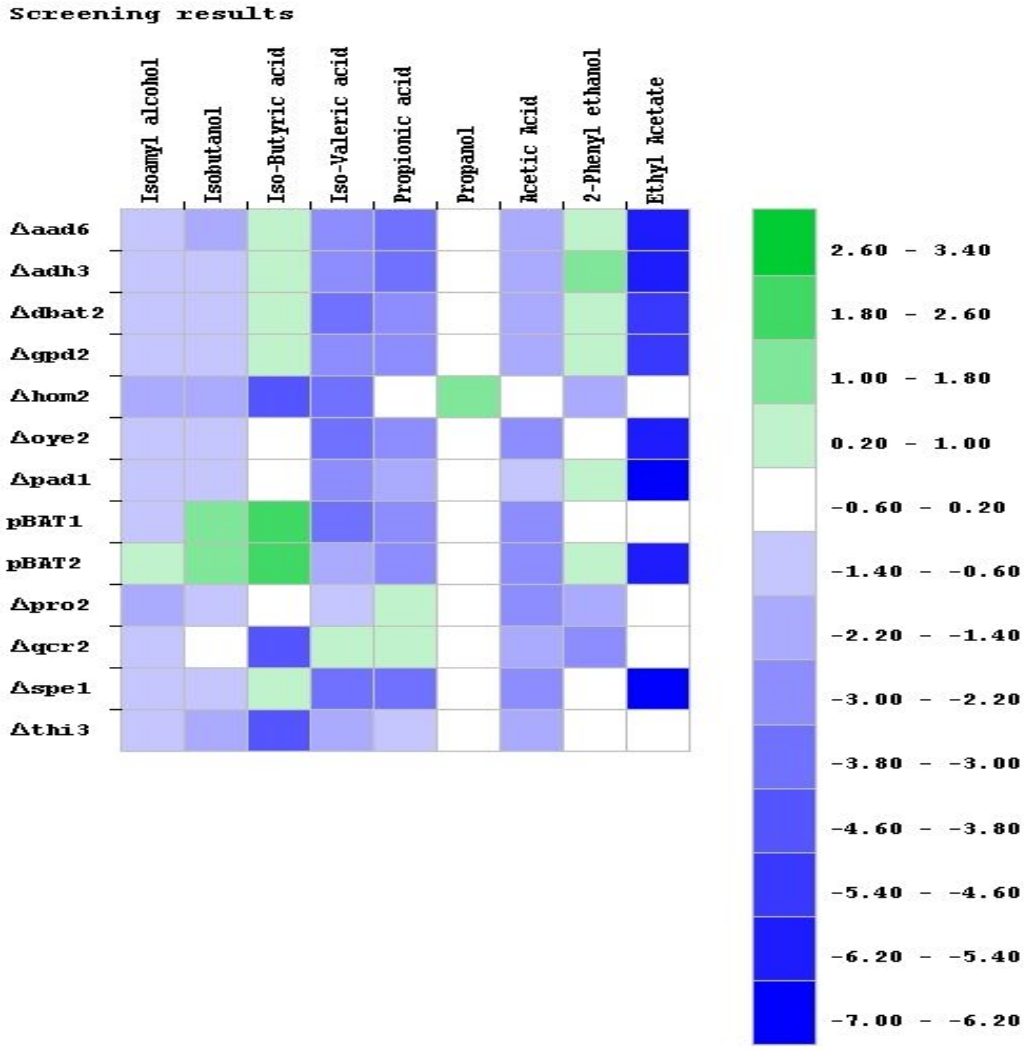


**Fig. 4.4.** Heat-map of the GC data of the 10 candidate strains, as well as certain control strains, indicating the levels of metabolites with regards to the wild type strain as the log2 of their ratio. An increase in the production of a certain metabolite is indicated by a green square and the intensity of the colour represents the fold value as indicated by the scale on the right hand side. A decrease is represented by a blue square.

The resulting comparisons between the EUROSCARF strains and independently deleted strains can be seen in **Figure 4.5**. The EUROSCARF strains are denoted by the capitalised letters, whilst the independent strains are notated in italicised small-case. The bars in **Figure 4.5** indicate the contribution of each compound to the total aroma profile of the strain and as such it is easy to compare the distribution of certain aroma compounds across different strains. It is clear that although there are slight differences between the strains in the final yield of certain aroma compounds, the higher alcohols and volatile fatty acids show good correlation between

**Table 4.4.** Gas Chromatographic (GC) data for deletion strains and control over-expression strain grown for one week in SCD5 medium. The results are the average of three replicates ± the standard error.

| SCD5 | | | | | | mg/l | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ID | 2-Phenyl Ethanol | Acetic Acid | Butanol | Ethyl Acetate | Hexyl Acetate | Isoamyl alcohol | Isobutanol | Iso-Butyric Acid | Iso-Valeric Acid | Propionic Acid |
| *Δ aad6* | 16.75±3.04 | 116.92±66.24 | 0.99±0.27 | 5.37±1.08 | 1.84±0.28 | 49.67±6.48 | 52.13±2.33 | 1.26±0.41 | 0.52±0.07 | 3.94±1.38 |
| *Δadh3* | 16.27±5.66 | 66.07±15.12 | 0.91±0.02 | 4.78±0.54 | 1.61±1.28 | 57.17±11.57 | 108.49±40.07 | 1.77±0.61 | 0.64±0.29 | 4.1±1.14 |
| *dbat1* | 10.24±0.01 | 28.89±0.14 | 0.92±0 | 4.34±0.09 | 1.4±0.01 | 39.11±0.78 | 27.82±0.38 | 0.57±0 | 0.24±0 | 2.07±0.03 |
| *dbat2* | 11.37±1.79 | 64.7±36.29 | 0.89±0.13 | 3.7±1.29 | 1.19±0.61 | 39.36±4.49 | 48.36±10.68 | 0.7±0.13 | 0.37±0.17 | 2.36±0.55 |
| *Δgpd2* | 17.55±7.22 | 1936.04±18.97 | 1.44±0.31 | 7.47±5.58 | 9.7±8.7 | 41.51±2.59 | 50.81±13.72 | 2.76±0.44 | 0.5±0.16 | 3.15±0.46 |
| *Δhom2* | 8.7±0.5 | 90.29±92.88 | 0.81±0.22 | 3.68±0.71 | 1.39±0.2 | 31.19±1.37 | 36.5±4.28 | 0.69±0.21 | 0.31±0.1 | 2±0.66 |
| *Δoye2* | 13.68±3.76 | 1883.75±4.53 | 1±0 | 6.87±4.47 | 8.69±5.04 | 39.03±8.99 | 75.3±14.93 | 2.32±0.22 | 0.47±0.25 | 5.62±1.92 |
| *Δpad1* | 9.38±0.83 | 1047.83±949.38 | 0.95±0.25 | 4.32±1.63 | 2.56±1.72 | 34.59±18.89 | 42.92±8.81 | 0.96±0.3 | 0.38±0.06 | 2.56±0.82 |
| *pBAT1* | 12.28±3.21 | 237.09±186.86 | 1.05±0.21 | 5.66±0.71 | 3.01±1.95 | 55.08±4.24 | 72.88±10.56 | 1.14±0.09 | 0.42±0.09 | 4.1±0.63 |
| *pBAT2* | 15.34±1.69 | 61.82±44.2 | 1.45±0.67 | 4.69±1.63 | 1.48±1.09 | 79.29±5.87 | 159.53±54.6 | 2.53±1.04 | 0.57±0.09 | 3.07±1.62 |
| *Δpro2* | 12.29±3.53 | 120.96±65.61 | 0.94±0.15 | 4.28±1.72 | 1.68±0.85 | 44.72±5.72 | 50.22±16.99 | 0.93±0.38 | 0.43±0.21 | 2.97±0.39 |
| *Δqcr2* | 10.46±2.04 | 85.52±58.74 | 0.85±0.15 | 4.1±0.72 | 0.89±0.78 | 44.18±6.5 | 41.36±5.6 | 0.88±0.39 | 0.44±0.26 | 2.36±1.19 |
| *Δspe1* | 12.47±4.01 | 662.25±832.64 | 0.93±0.61 | 6.23±4.35 | 6.79±8.28 | 37.78±2.58 | 45.6±10.75 | 1.54±1.18 | 0.42±0.15 | 2.64±0.76 |
| *Δthi3* | 20.4±5.48 | 545.96±941.9 | 0.62±0.56 | 3.78±5.57 | 0.59±0.76 | 47.29±7.75 | 47.57±13.09 | 2.91±1.58 | 0.99±0.26 | 5.31±3.6 |
| *WT* | 17.12±3.78 | 1378.71±1450.33 | 1.29±0.33 | 7.96±1.37 | 4.05±3.44 | 69.71±9.3 | 116.81±35.74 | 1.36±0.59 | 1.08±0.6 | 8.1±5.58 |

**Table 4.5.** GC data for deletion strains and control over-expression strain grown for one week in SCD5+++ medium. The results are the average of three replicates ± the standard error.

| SCD5+++ | | | | | | | mg/l | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 2-Phenyl Ethanol | Acetic Acid | Decanoic Acid | Ethyl Acetate | Hexyl Acetate | Isoamyl alcohol | Isobutanol | Iso-Butyric Acid | Iso-Valeric Acid | Octanoic Acid | Propanol | Propionic Acid |
| Δ*aad6* | 48.81±3.39 | 2195.99±220.48 | 0.63±0.29 | 13.56±4.1 | 14.42±3.53 | 49.23±2.81 | 66.81±16.87 | 7.55±0.31 | 1.37±0.07 | 1.5±0.14 | 34.24±8.06 | 9.77±1.23 |
| Δ*adh3* | 43.64±6.66 | 2299.84±533.44 | 0.5±0.35 | 14.56±4.43 | 10.36±2.35 | 54.32±8.39 | 81.82±21.56 | 8.85±1.16 | 1.1±0.16 | 1.45±0.44 | 36.97±8.31 | 8.45±0.8 |
| *dbat1* | 50.68±5.66 | 2322.82±104.11 | 0.78±0.29 | 14.61±1.95 | 14.01±3.27 | 54.86±5.75 | 79.78±13.55 | 8.35±0.6 | 1.5±0.1 | 1.52±0.3 | 34.88±5.69 | 9.37±1.54 |
| *dbat2* | 52.64±2.57 | 2355.69±163.3 | 0.63±0.37 | 16.26±2.53 | 16.54±2.68 | 38.89±4.4 | 54.33±8.89 | 5.07±0.22 | 0.92±0.06 | 1.44±0.21 | 37.28±6.37 | 10.54±0.45 |
| Δ*gpd2* | 54.03±2.35 | 2418.97±110.33 | 0.9±0.22 | 18±0.74 | 14.75±2.6 | 65.92±5.41 | 85.7±4.86 | 10.06±1.11 | 1.9±0.21 | 1.94±0.48 | 41.24±3.07 | 9.56±0.31 |
| Δ*hom2* | 21.56±0.48 | 1210.63±866.87 | 0.85±0.23 | 7±0.83 | 31.7±2.42 | 32.45±4.53 | 69.14±7.23 | 8.09±1.7 | 1.11±0.15 | 2.38±0.45 | 14.11±0.85 | 3.39±0.47 |
| Δ*oye2* | 41.6±5.35 | 2036.69±93.6 | 0.85±0.47 | 13.11±0.79 | 8.42±1.79 | 50.71±5.78 | 69.28±6.69 | 6.9±0.63 | 1.04±0.12 | 2.09±0.15 | 34.1±4.36 | 7.07±0.47 |
| Δ*pad1* | 50.3±2.41 | 2397.59±35.43 | 0.86±0.13 | 13.78±2.62 | 13.4±2.93 | 55.22±5.04 | 74.94±8.64 | 8.62±0.69 | 1.61±0.13 | 1.92±0.19 | 35.67±5.12 | 10.4±1.11 |
| *pBAT1* | 48.61±2.2 | 2299.95±81.98 | 1.23±0.86 | 12.37±1.23 | 11.54±0.73 | 46.53±5.23 | 153.6±13.19 | 15.49±0.86 | 1.36±0.07 | 1.5±0.82 | 33.36±2.93 | 8.74±0.69 |
| *pBAT2* | 32.07±1.9 | 2387.1±208.54 | 1.05±0.25 | 14.54±2.76 | 10.64±3.43 | 91.08±5.96 | 351.13±49.34 | 40.97±0.49 | 2.54±0.1 | 2.12±0.04 | 34.21±6.2 | 7.36±1.04 |
| Δ*pro2* | 107.01±5.21 | 426.44±62.76 | 0.22±0.09 | 7.72±1.95 | 6.76±0.74 | 81.52±4 | 76.88±15.81 | 12.8±2.03 | 4.24±0.08 | 0.57±0.11 | 35.01±8.04 | 11.48±1.13 |
| Δ*qcr2* | 20.93±1.22 | 856.8±24.11 | 1.02±0.18 | 9.9±2.44 | 0.21±0 | 51.57±2.64 | 92.93±23.65 | 13.62±1.69 | 1.63±0.08 | 2.47±0.84 | 22.73±5.22 | 3.16±0.44 |
| Δ*spe1* | 49.52±6.13 | 2256.75±242.82 | 0.84±0.15 | 15.33±2.92 | 15.48±4.13 | 55.46±6.73 | 76.1±13.96 | 8.65±1.05 | 1.6±0.21 | 1.69±0.28 | 37.44±7.92 | 9.69±0.53 |
| Δ*thi3* | 46.29±3 | 1957.87±197.4 | 0.73±0.15 | 9.57±3.36 | 7.27±2.17 | 52.54±3.27 | 56.92±3.27 | 7.87±0.72 | 1.48±0.19 | 1.72±0.22 | 21.95±5.18 | 8.1±0.83 |
| *WT* | 63.5±10.4 | 3023.37±78.34 | 8.39±6.17 | 15.9±0.12 | 15.6±0.07 | 81.42±2.85 | 99.84±1.2 | 8.88±3.89 | 2.18±0.5 | 3.59±1.01 | 37.93±14.18 | 7.93±4.55 |

independent- and EUROSCARF deletion strains, with the exception of Δ*qcr2*. It is also important to note that these results have not been corrected for growth differences and thus the differences seen in **Figure 4.5** might be simply due to growth effects and not the manner in which the gene has been deleted.

In all cases, except in the case of Δ*qcr2*, the results confirmed those obtained with the EUROSCARF strains, both from a quantitative and qualitative perspective. This indicates that the QCR2 deletion strain in the EUROSCARF library either carries some secondary mutations that affect aroma production, or that the deletion of QCR2 has some impact on neighbouring genes. As seen with the EUROSCARF deletion library, deletion of *HOM2* had the most important effect on the final levels of both isoamyl alcohol and isobutanol, as well as on the concentrations of iso-butyric and iso-valeric acid.

In order to further verify that the candidate genes indeed influence the metabolites and end products of the Ehrlich pathway in fermentative conditions, the deletion strains were reassessed in small scale batch fermentations with a glucose concentration of 5% (w/v), and strains were grown in medium with (SCD5+++) and without (SCD5) added amounts of the three branched-chain amino acids; leucine, isoleucine and valine. All fermentations were carried out in triplicate.

Results confirmed the data from the large scale screening, i.e. that the deletion of the genes in each case led to a decrease in the amounts of higher alcohols and related compounds formed during the fermentation (**Tables 4.4** and **4.5**). The data also showed that all strains continued to show fermentation kinetics similar to the WT, suggesting that the observed effects were not due to some indirect impact on growth (supplemental material). The statistical analysis of the data, in the form of p values can be seen in **Table 4.6**.

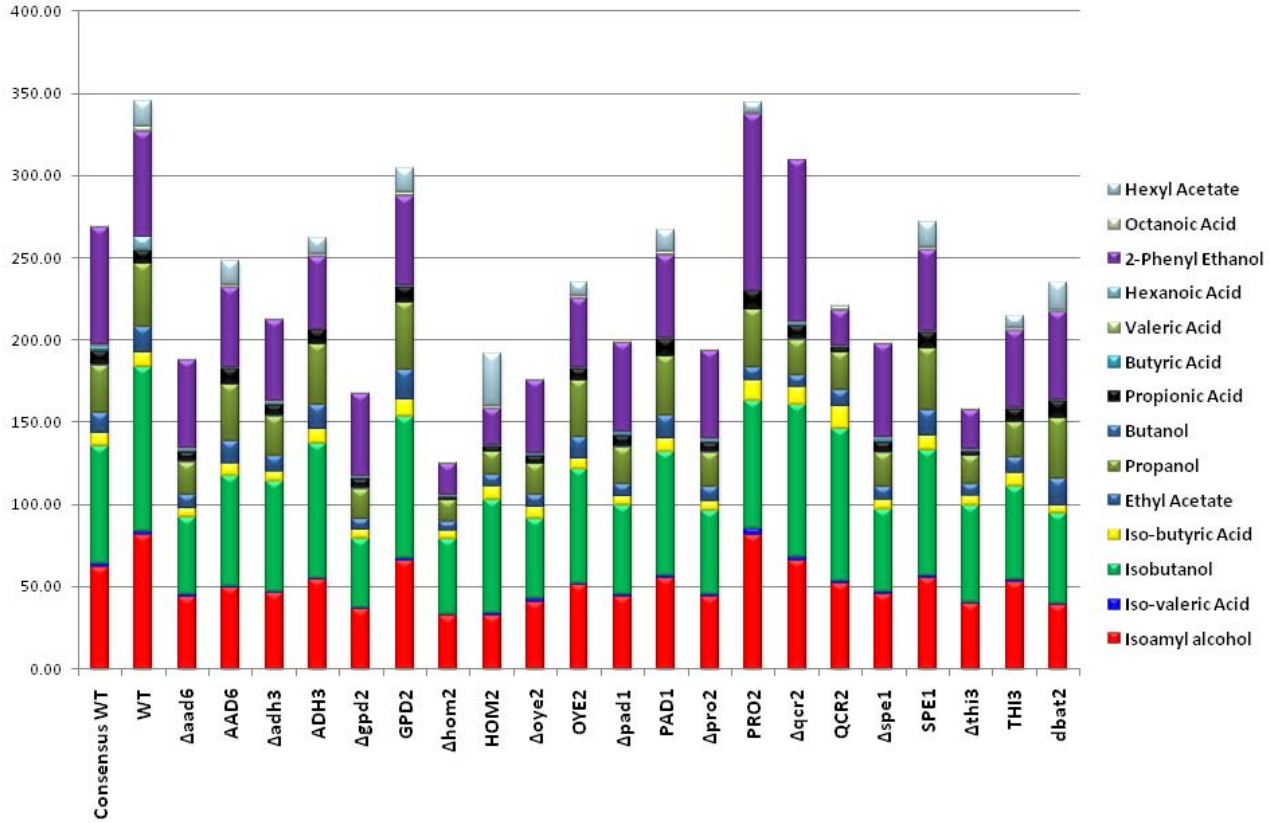


**Fig. 4.5.** Stack plots of the total aroma profile of independently deleted and EUROSCARF deletion strains, with the contribution of each of the aroma compounds indicated in the legend, represented by the size of the coloured bar in the stack. EUROSCARF strains are denoted by the capitalised letters, whilst the independent strains are notated in italicised small-case.

In **Figure 4.6** the final concentrations of the end products of the Ehrlich pathway for the deletion strains grown in SCD5 and SCD5+++ medium can be seen. Once again it is clear from **Figure 4.6** (top panel) that the over-expression of the *BAT2* gene leads to a 1.3 fold increase in the level of isobutanol produced. It is interesting to note that in this instance the effect on the concentration of isoamyl alcohol is much less pronounced than in the initial screen where the total glucose concentration in the medium was 2 g/l (w/v), compared to the SCD5 medium with a higher glucose concentration of 5 g/l (w/v). It is possible that the higher glucose concentration, might suppress the catabolism of specific branched-chain amino acids to some extent.

**Table 4.6.** Results of statistical analysis of a section of the gas chromatographic data with Student's t-test. A p value smaller than 0.05 is deemed significant.

| | p Value | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | SCD5 medium | | | | SCD5+++ medium | | | |
| | Isoamyl alcohol | Isobutanol | Iso-Butyric Acid | Iso-Valeric Acid | Isoamyl alcohol | Isobutanol | Iso-Butyric Acid | Iso-Valeric Acid |
| Δ*aad6* | 0.0001 | 0.0006 | 0.7095 | 0.0228 | 1.8414E-08 | 0.0020 | 0.4872 | 0.0219 |
| Δ*adh3* | 0.0226 | 0.6484 | 0.1978 | 0.0688 | 0.0001 | 0.0959 | 0.9858 | 0.0068 |
| *dbat1* | 7.59E-06 | 0.0001 | 0.0122 | 0.0030 | 7.69E-08 | 0.0021 | 0.7768 | 0.0369 |
| *dbat2* | 1.72E-06 | 0.0003 | 0.0259 | 0.0075 | 3.2163E-10 | 0.0000 | 0.0932 | 0.0047 |
| Δ*gpd2* | 7.21E-06 | 0.0004 | 0.0005 | 0.0212 | 0.0053 | 0.0084 | 0.5473 | 0.3065 |
| Δ*hom2* | 1.23E-06 | 0.0001 | 0.0244 | 0.0046 | 8.05E-11 | 0.0001 | 0.9059 | 0.0068 |
| Δ*oye2* | 4.91E-05 | 0.0096 | 0.0041 | 0.0216 | 1.61E-08 | 1.19E-05 | 0.3201 | 0.0060 |
| Δ*pad1* | 0.0003 | 0.0002 | 0.1447 | 0.0081 | 6.27E-07 | 0.0002 | 0.8900 | 0.0620 |
| *pBAT1* | 0.0014 | 0.0061 | 0.3624 | 0.0106 | 9.66E-06 | 0.0008 | 0.0175 | 0.0209 |
| *pBAT2* | 0.0209 | 0.0700 | 0.0138 | 0.0346 | 0.0015 | 3.28E-07 | 4.54E-05 | 0.1898 |
| Δ*pro2* | 1.01E-05 | 0.0003 | 0.1248 | 0.0118 | 0.9617 | 0.0084 | 0.0889 | 0.0007 |
| Δ*qcr2* | 8.26E-06 | 0.0002 | 0.0917 | 0.0133 | 5.35E-08 | 0.4362 | 0.0501 | 0.0676 |
| Δ*spe1* | 3.15E-06 | 0.0002 | 0.7003 | 0.0112 | 2.04E-06 | 0.0019 | 0.9030 | 0.0580 |
| Δ*thi3* | 4.85E-05 | 0.0003 | 0.0206 | 0.6747 | 8.15E-08 | 3.16E-08 | 0.5956 | 0.0322 |

All the deletion strains, except Δ*adh3*, show a decrease in the levels of both isoamyl alcohol and isobutanol when compared to the wild type yeast strain when grown in SCD5 medium. These decreases are once again in the same range, or lower than the decreases seen with the *dbat2* strain. This strongly suggests that these genes, with the possible exception of Δ*adh3*, impacts on the conversion of the branched-chain amino acids into higher alcohols in fermentative conditions.

As expected, the level of isobutanol produced by the over-expression strain is increased tremendously when grown in branched-chain amino acid enriched medium, SCD5+++, although once again this effect was not seen with isoamyl alcohol. However, all the deletion strains showed decreases in the final concentrations of these two higher alcohols, with the Δ*hom2* deletion giving the biggest effect.

The scenario with the volatile fatty acids is less clear. In the SCD5 medium (**Figure 4.6**, bottom panel) the *BAT2* over-expression strain, as well as the strains Δ*adh3*, Δ*gpd2*, Δ*oye2*, Δ*spe1* and Δ*thi3* all show an increase in the levels of iso-butyric acid. The concentrations of iso-butyric acid are reduced in the remainder of the deletion strains. In the case of iso-valeric acid, however, all the deletion strains except for Δ*thi3* show a marked drop in the levels produced.

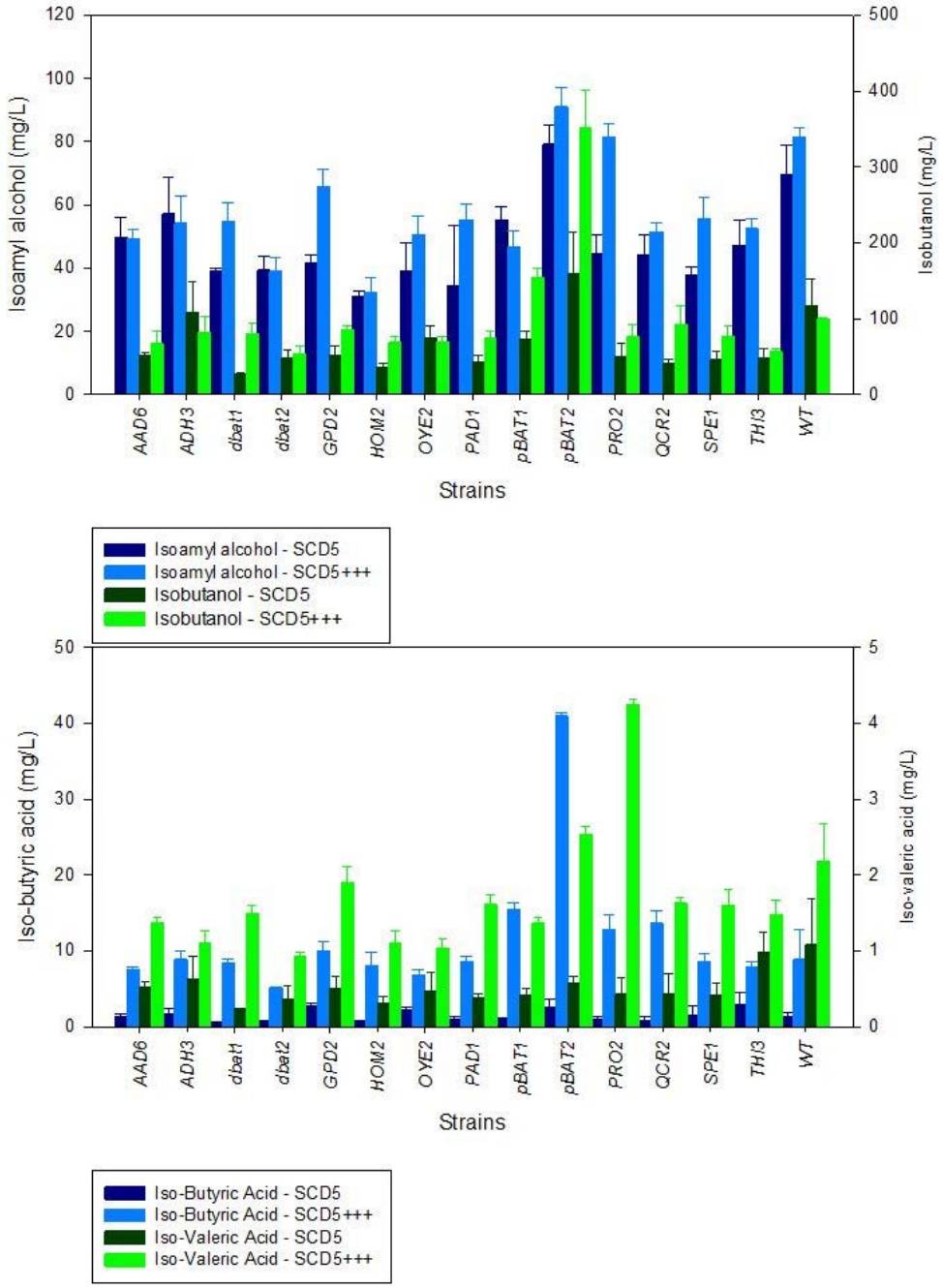


**Fig. 4.6.** GC-FID analysis of the concentrations of the two major higher alcohols formed from the branched-chain amino acids, isoamyl alcohol and isobutanol, as well as two major higher volatile fatty acids, iso-butyric and iso-valeric acid, formed after cells were grown for one week in SCD5 or SCD5+++ medium. The graph shows the differences between the deletion strains, certain control strains and the wild type. In the top panel, dark blue bars indicate concentrations of isoamyl alcohol formed from cells grown in SCD5 medium and light blue bars the concentration of isoamyl alcohol from SCD5+++ grown strains. The dark and light green bars indicate the same differences in growth medium for levels of isobutanol produced. In the bottom panel, dark blue bars indicate concentrations of iso-butyric acid formed from cells grown in SCD5 medium and light blue bars the concentration of iso-butyric acid from SCD5+++ grown strains. The dark and light green bars indicate the same differences in growth medium for levels of iso-valeric acid produced. The experiments were performed in triplicate and the error bars are shown.

Of the strains grown in the SCD5+++ medium (**Figure 4.6**, bottom panel), the *BAT1* and *BAT2* over-expression strains, as well as Δ*gpd2*, Δ*pro2*, and Δ*qcr2* have elevated iso-butyric acid levels when compared to that of wild type strain. All the other deletion strains show a decrease in the concentration of this compound. The effect on the production of iso-valeric acid is much simpler, with all the deletion strains, except Δ*pro2* giving less or similar levels than for the wild type strain.

### 4.4.3 THE EFFECT OF GENE DELETION ON THE PRODUCTION OF OTHER AROMA RELATED METABOLITES

From previous work where the BAT genes were perturbed [22] it was observed that not only were concentrations of metabolites directly linked to these genes affected, but also other aroma metabolites not directly related to the higher alcohols. These results lead to speculation on the interconnectedness of the aroma compound production pathway in *Saccharomyces cerevisiae*. We also wanted to investigate this aspect in the present study.

A metabolic map that links all metabolites listed in **Tables 4.4** and **4.5** to the branched-chain amino acid metabolism was developed to analyse the effects of the deletion of the candidate genes on other aroma-related compounds. A simplified diagram of this metabolic map is shown in **Figure 4.1**. This metabolic map was used to generate schematic diagrams of the changes in aroma profile due to gene deletion. The ratio of all measured aroma compound from wild type compared to the deletion strain were calculated and transformed by log2 to ensure an even spread of the fold differences. These values were then represented in the thickness of the arrow as seen in **Figure 4.7**. Only the most relevant strains are shown in **Figure 4.7**.

As Gpd2p is unlikely to play a direct role in the Ehrlich pathway, the effects caused by the deletion of the *GPD2* gene on the aroma profile is likely due to be indirect and linked to the maintenance of redox homeostasis. That the aroma profile is in any case affected by the deletion of the *GPD2* gene

shows that the yeast aroma production pathway, and especially the Ehrlich pathway are indeed sensitive to redox homeostasis.

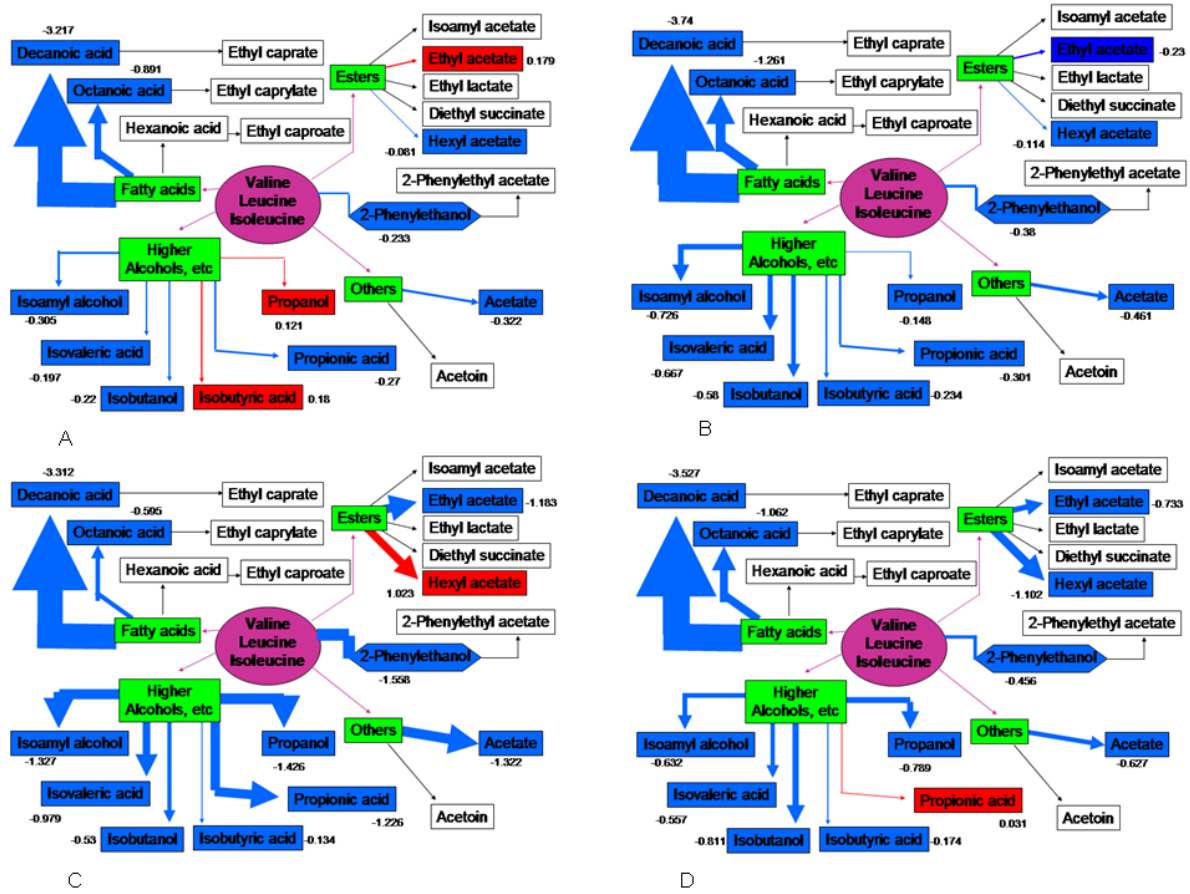


**Fig. 4.7.** Schematic representation of the effect of certain selected gene deletions on the yeast aroma profile in cells grown in SCD5+++ medium. Red indicates an increase and blue a decrease, whilst the arrow thickness gives an indication of the magnitude of the effect in log2. The panels correspond to the following deletion strains, A = Δ*gpd2*, B = Δ*aad6*, C = Δ*hom2*, D = Δ*thi3*

The effect of the Δ*gpd2* strain on the aroma profile is seen in **Figure 4.7**, panel A, together with three strains that impacted the most on the Ehrlich pathway end products. Deletion of these three genes – *AAD6* (panel B), *HOM2* (panel C) and *THI3* (panel D) – are unlikely to have any impact on the global redox balance of the cell and therefore their impact on the Ehrlich pathway is much more likely to be a direct one. It is also interesting to note that deletion of all four genes had a huge negative impact on the concentration of decanoic acid and to a lesser extent that of octanoic acid.

Acetate production plays an important role in cellular redox maintenance. The effect of gene deletion on the production of this compound is very much dependant on medium composition with decreases in concentration seen in strains grown in the SCD5 medium (**Table 4.4**). In line with the role of acetate production, the levels of this compound are affected by the deletion of reductases and dehydrogenases.

**Figure 4.7** shows that only two esters are affected by the deletions of the selected genes, i.e. ethyl acetate and hexyl acetate. These acetate esters are synthesized by acetyltransferase enzymes which react with acetyl coenzyme A (acetyl-CoA), derived from either pyruvate, acetaldehyde or acetate, and, depending on the degree of affinity, with various higher alcohols to produce esters [44]. In most cases the concentrations of these esters are negatively influenced by gene deletion, the only significant exception being the slight increase of ethyl acetate seen in the Δ*gpd2* strain. The decrease in concentration of these two acetate esters can be directly linked to the phenomenon of decreased acetate production, discussed above. Less acetate would lead to less acetyl-CoA being present and thus to less esters being formed.

Another compound that is affected by the deletion of the selected genes (as seen in **Figure 4.7**) and that also plays an important part in the aroma profile is 2-phenylethanol. This higher alcohol is formed from the aromatic amino acid phenylalanine, also via the Ehrlich pathway. Previous work [33-35] showed that various genes can catalyze the conversion of aromatic amino acids into higher alcohols, including some involved in this study. It is thus likely that a deletion that has an effect of decreasing the concentrations of branched-chain amino acid derived higher alcohols, would also decrease the concentrations of other compounds that are formed similarly.

### 4.4.4 MULTIVARIATE DATA ANALYSIS

Multivariate analysis of the dataset in the form of principle component analysis (PCA) was performed in order to investigate the factors contributing to the variance between the 10 candidate genes and the control strains. The first and second components, PC1 and PC2, were able to explain 33% and 22%, respectively, of the observed variance between the deletion strains. **Figure 4.8**, top panel, illustrates the bi-plot of PC1 *vs.* PC2 and show that the deletion strains are spread out along the first component axis, with the two over-expression strains, *pBAT1* and *pBAT2* clustering together towards PC2, with the compounds most involved in explaining this distance to the other strains being: isobutanol, iso-butyric acid and isoamyl alcohol.

A closer look along the PC1 axis shows that the three deletion strains Δ*aad6*, Δ*adh3* and Δ*gpd2* formed a distinct cluster. The compounds most responsible for this cluster are 2-phenyl ethanol, butyric acid, hexanoic acid and acetate (**Figure 4.8**, middle panel). *ADH3* and *GPD2* are major regulators of redox balancing under fermentative conditions, the fact that Δ*aad6* clusters together with such genes suggests that this gene plays a direct role in the Ehrlich pathway.

**Figure 4.8** also shows that compounds such as propanol, valeric acid, diethyl succinate, iso-valeric acid and propionic acid are important in explaining the difference between Δ*thi3* and Δ*hom2* and the other selected mutants. It is interesting that these two genes cluster close to one another as they seem to have the biggest impact on higher alcohol and related metabolite production of the 10 selected genes are likely to play a direct role in the Ehrlich pathway. The distant cluster of Δ*qcr2* and Δ*pro2* might be explained by the fact they are the only two reductases included in the 10 candidate genes and therefore are completely different from the rest of the group. It is thus unlikely that they could play a direct part in the metabolism of the higher alcohols and volatile branched-chain fatty acids.

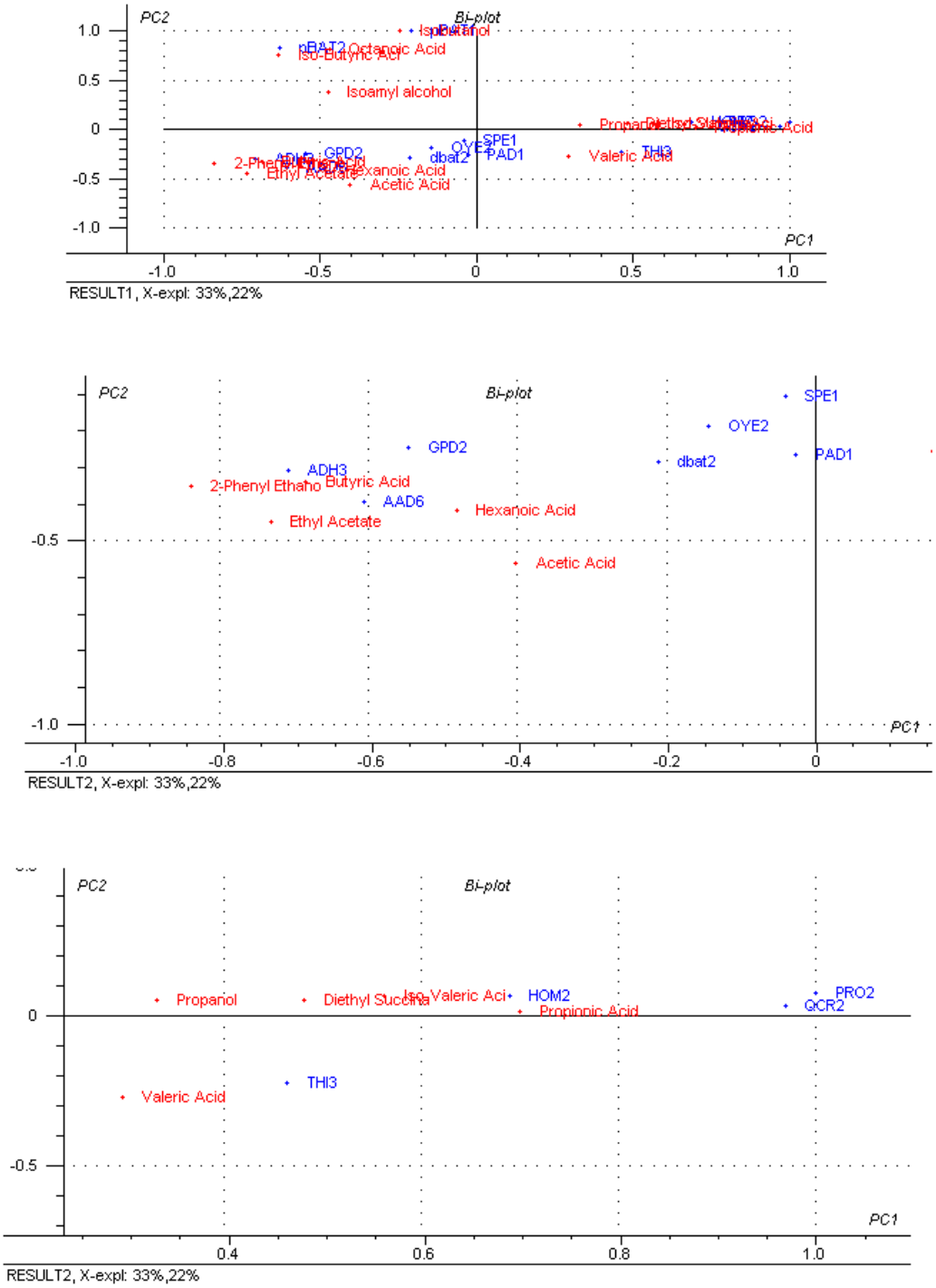


**Fig. 4.8**. Bi-plot of component 1 vs. component 2 of a principal component analysis of the gas chromatographic dataset of the ten candidate genes as well as various control genes. The top panel shows the bi-plot in totality, whilst the middle panel focuses on the left side of PC1 and the bottom panel focuses on the right hand side of PC1

## 4.5 DISCUSSION

From the above evidence it is clear that deletion of the selected genes has a significant impact on the catabolism of branched-chain amino acids via the Ehrlich pathway into higher alcohols and volatile acids, as well as the general aroma profile of the yeast strain. However, it is also clear that not all genes are directly involved in these chemical reactions and many exert their effect via indirect means, with the maintenance of redox homeostasis being the most obvious role player. Indeed, two of the genes are involved in major redox maintenance by regenerating $NAD^+$ during fermentative conditions. *GPD2* encodes for the NAD-dependent glycerol 3-phosphate dehydrogenase located in the cytosol and mitochondrion [45]. Gpd2p catalyzes the conversion of dihydroxy-acetone phosphate to glycerol-3-phosphate with the simultaneous oxidation of NADH to $NAD^+$ [46-49]. *ADH3* encodes for the mitochondrial alcohol dehydrogenase isozyme III [50]. Adh3p is involved in the NADH-dependent reduction of acetaldehyde to ethanol during glucose fermentation and plays a part in the ethanol-acetaldehyde redox shuttle that helps in maintaining the redox balance of the mitochondrion [51, 52]. Previous authors [32] investigated whether *ADH3* can play a role in the formation of higher alcohols, and indeed found that any of the ADH genes can fulfil this function. Interestingly in this study no significant effect was seen on higher alcohol production when any of the other ADH genes were deleted. The results clearly demonstrate that the Ehrlich pathway is highly sensitive to the general redox balance of the cell, and that the redox duality of the pathway can be useful in overcoming perturbations caused by the deletion of important redox regulating genes. In addition, the data suggest that the other *ADH* genes do not normally play a direct role within this pathway.

Three other dehydrogenases were also amongst the selected genes investigated. *OYE2* is a well conserved NADPH dehydrogenase, but its exact function is still unclear [53-56]. It is able to oxidize both NADPH and NADH, although less efficiently, and various phenolic compound may be able to bind to it [57, 58]. Other researchers have found that Oye2p can associate with actin and protect

the actin cytoskeleton from oxidative stress [59]. However, a study that impacts on the results from this study found that Oye2p can reduce acrolein and other α,β-unsatturated aldehydes [60]. This might implicate *OYE2* to a direct role in the Ehrlich pathway. Indeed deletion of *OYE2* leads to notable decreases in the levels of isoamyl alcohol, isobutanol and iso-valeric acid, but large increases in iso-butyric acid concentrations was sometimes observed. It would seem that the cell has to maintain a fine balance between redox maintenance and the removal of the potentially toxic aldehydes formed during the Ehrlich reaction.

The data suggest that the other two dehydrogenase genes may be directly involved in the Ehrlich pathway. *AAD6* is a putative aryl-alcohol dehydrogenase with high sequence homology to the lignin degrading fungus *Phanerochaete chrysosporium* [61, 62]. During the breakdown of lignin by this white rot fungus, various peroxidases are very active, leading to products with many aldehyde-, quinine- and acidic groups which must be reduced by the *AAD* gene products [63]. However, while *S. cerevisiae* contains seven *AAD* genes [62], aryl-alcohol dehydrogenase activity found in this yeast appears to be independent of these genes and their substrate is still unknown [61]. Dickinson, *et al*. (2003), speculated that these genes may be involved in the Ehrlich pathway, but studies with a strain deleted in all seven *AAD* genes showed no effect on the level of isoamyl alcohol produced [32]. This is in contrast to the present study which shows decreases in the level of isoamyl alcohol, isobutanol and iso-valeric acid. These contradictory findings may be explained by the composition of the medium, Dickinson *et al*. (2003) used a minimal medium with only leucine, whilst our media contained all amino acids. Furthermore, our data strongly suggest that of the seven *AAD* genes, *AAD6* is the main enzyme involved in the pathway.

Deletion of the *HOM2* gene leads to the greatest reduction in the concentration of higher alcohols and volatile acids, suggesting that *HOM2* plays a central and direct role in the formation of both the higher alcohols and volatile acids. The role of this enzyme in the Ehrlich pathway would be the oxidation or reduction of the preceding aldehyde to from either a branched-chain fatty acid or a

higher alcohol. *HOM2* encodes for an aspartic beta semi-aldehyde dehydrogenase, which catalyzes the second step in the pathway for methionine and threonine biosynthesis. This step involves the reduction of aspartyl-4-phosphate to aspartate-4-semialdehyde, using NADPH [64, 65]. However, when de Robichond-Szulmajster *et al*. (1966) measured the enzymatic activities of this enzyme, they did so in the reverse direction [66], giving credence to the direct role that this enzyme plays in the final reaction of the Ehrlich pathway and the ability of this enzyme to reduce or oxidise the aldehyde precursor. This also explains the dramatic negative effects seen on the concentrations of both higher alcohols and volatile branched-chain fatty acid when the gene is deleted.

Independent deletions of the *QCR2* gene could not repeat the effects of the EUROSCARF deletion of this gene on higher alcohol production indicating an erroneous identification during the screening process and an unlikely role for *QCR2* in the Ehrlich pathway. *PRO2* deletion leads to some contradictory results regarding the production of these compounds. *PRO2* encodes for a gamma-glutamyl phosphate reductase and catalyzes the second step in proline biosynthesis [67]. More specifically it is responsible for the conversion of γ-Glutamyl phosphate and NADPH into L-Glutamate-γ-semialdehyde and $NADP^+$ [68].

The last three genes are all classified as decarboxylases and could catalyse the conversion of the α-keto acid into its corresponding aldehyde in the Ehrlich reaction. *PAD1* and *SPE1* are both thought to encode decarboxylases that can act promiscuously and accept various chemical compounds as substrates. The gene product of *PAD1* is a phenylacrylic acid decarboxylase and confers resistance to cinnamic acid and other phenylacrylic acids [69]. Pad1p has also been shown to decarboxylate phenolic or aromatic carboxylic acids to their corresponding vinyl derivatives [70]. *SPE1* encodes an ornithine decarboxylase which catalyzes the first step in polyamine biosynthesis in the cytoplasm, more specifically the decarboxylation of ornithine into putrescine [71-73]. It is degraded in a proteasome-dependent manner in the presence of excess polyamines [74-76]. Other

authors have recently shown that the enzymes that decarboxylate aromatic amino acids, i.e. *ARO10*, can play a role in the Ehrlich pathway and may also decarboxylate non-aromatic amino acids [34]. This might indicate that Pad1p and Spe1p display similar promiscuity towards their substrate and can directly decarboxylate the α-keto acids formed after transamination of the branched-chain amino acids.

The last gene identified in the screening procedure has previously been suggested to be involved with the Ehrlich pathway. *THI3* (also known as *KID1*) encodes a probable decarboxylase which plays a role as a regulatory protein of the enzymes involved in thiamine biosynthesis [77-79]. Thi3p may also play a role in the catabolism of amino acids to long-chain and complex alcohols [29, 32]. This last scenario fits well with the data from this study and the results suggest that *THI3* indeed plays a direct role in the Ehrlich pathway

In summary, this study identified a set of genes with direct and indirect impacts on the Ehrlich pathway and the aroma profile in general. The genes *ADH3* and *GPD2* are well known role players in cellular redox maintenance and as such are unlikely to play a direct role in the Ehrlich pathway. Rather effects seen when these genes are deleted are due to perturbations of the cellular redox homeostasis and indicate that the Ehrlich pathway, as well as other pathways of aroma production, are sensitive to changes in cellular redox homeostasis. Genes such as *AAD6*, *HOM2* and *THI3*, however, are unlikely to affect cellular redox maintenance to the same extent as the above genes, but deletion of these three genes had an even greater impact on the Ehrlich pathway – indicating that these genes play a direct role in the Ehrlich pathway. Lastly, the remaining candidate genes, *OYE2*, *PAD1*, *PRO2*, *QCR2* and *SPE1* had varying levels of effect on the Ehrlich pathway and analysis of the data indicates that these genes could function as promiscuous genes with a wide substrate range. However, further studies are needed before the above can be taken as fact.

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## 4.7 SUPPLEMENTAL MATERIAL

### 4.8.1. FERMENTATION KINETICS OF THE DELETION STRAINS

In order to establish growth kinetics, strains were inoculated as stated in the Materials and Methods section, but OD samples were taken every 6 h for the first 24 h and thereafter less regularly. Fifty microlitres of the medium were added to 200 µl $H_2O$ in a 96-well plate, mixed by pipetting and read at 600 nm in a Powerwave X spectrophotometer (Bio-Tek Instruments, Inc.).

To ensure that the effects seen on aroma compound production, as discussed above, are in fact due to the deletion of the specific gene and not an indirect result of a diminished fermentation capacity, the fermentation kinetics of all ten strains in comparison with the wild type strain and the previously constructed *dbat2* strain were evaluated [22].

**Figure A1A** indicates that there are no differences in the fermentation rate and biomass production between the various deletion strains and the wild type and *BAT2* deletion strains. There are also no significant differences between the strains during the crucial first 48 h growth period, as shown in **Figure A1B** suggesting that the effects seen on the production of higher alcohols and related compounds is in fact due to the specific gene deletion in the strain and not the effect of diminished fermentation performance.

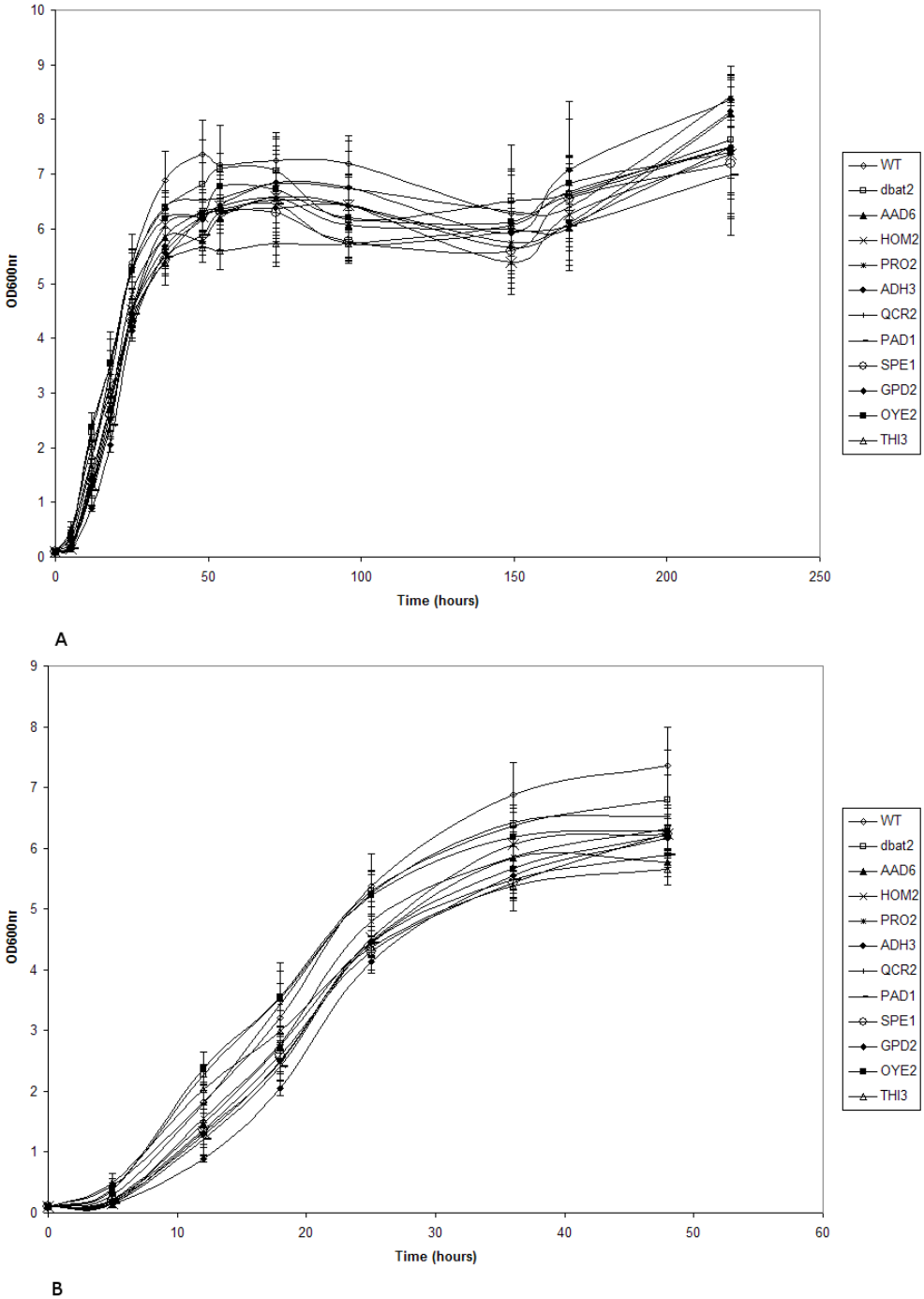



**Fig. A1**. The fermentation kinetics of the deletion strains compared to the wild type yeast strain, in the form of a growth curve. Panel A shows the complete growth curve over the space of 220 hours, whilst panel B shows the important first 48 hours in more detail.

# Chapter 5

# Research results

## Genetic analysis of the metabolic pathways responsible for higher alcohol production in *Saccharomyces cerevisiae*

**A modified version of this paper is currently being submitted to Applied and Environmental Microbiology**

# 5. GENETIC ANALYSIS OF THE METABOLIC PATHWAYS RESPONSIBLE FOR HIGHER ALCOHOL PRODUCTION IN *SACCAROMYCES CEREVISIAE*

## 5.1 ABSTRACT

During alcoholic fermentation, most higher alcohols are formed from amino acids via the Ehrlich pathway by yeast and impact on wine flavour and aroma. While the broad metabolic pathway of higher alcohol formation has been described, many of the individual genes encoding the enzymes involved in the pathway and its regulation have not been identified or elucidated, and the physiological role of higher alcohol formation remains unclear. Previous work showed that the gene products of *BAT1* and *BAT2* contributed significantly to the first step of the pathway, the deamination of amino acids, since deletion of these genes reduced significantly the formation of higher alcohols. By screening a set of yeast strains with deletions in genes encoding decarboxylases, dehydrogenases and reductases, we recently identified a number of genes whose absence impacted significantly on higher alcohol production. Ten genes, whose deletion impacted the most significantly on higher alcohol production were selected. Here we present an in-depth genetic analysis to further our understanding of the precise roles of these genes in the broader aroma production metabolic network by using over-expression and multiple deletion of genes.

## 5.2 INTRODUCTION

Yeast strains contribute significantly to the final aroma profile or bouquet of wine [1-4], and many studies have identified specific aroma compounds that are formed by this organism during the fermentation of grape must into wine. Such impact compounds include esters [5-7], volatile fatty acids [8-10] and higher alcohols [11-13]. These compounds not only play a crucial role in the formation of wine aroma [3, 14, 15], but also other fermented products such as beer [16, 17], cider [12] and cheese [18, 19].

While the metabolic network that leads to the formation of these compounds is reasonably well mapped, surprisingly little is known about specific enzymes involved in specific reactions, the genetic and metabolic regulation of the network and the physiological roles of individual pathways within the network. Furthermore, different yeast strains tend to produce significantly different aroma profiles, and aroma production capability is a major target of yeast strain improvement programs globally [4, 20].

Previous work (Styger *et al*., this dissertation), used a mutant screening approach to identify 10 genes that impacted most significantly on this metabolic network. The primary focus was directed towards elements that are involved in the conversion of branched-chain amino acids to aroma compounds, a pathway known as the Ehrlich reaction (**Figure 5.1**) [21]. Indeed, many data sets have confirmed that amino acids constitute the most relevant precursors for the biosynthesis of these volatile compounds [22]. In this study these selected genes are investigated further through the use of double and triple deletion mutants.

Branched chain amino acids are most directly linked to many of the volatile aroma compounds that are desired in wine. These branched-chain amino acids, leucine, isoleucine and valine, are transported into the yeast cell via four transport systems, the general amino acid permease Gap1p [23-27], the high affinity branched-chain amino acid permease Bap2p [28-33], the closely related branched-chain amino acid permease Bap3p [34-36] and the tyrosine transporter Tat1p [35]. These amino acids can be used in several ways. In particular, they can be used as such for protein synthesis, or metabolized into other compounds and used for other purposes and metabolic processes [37].

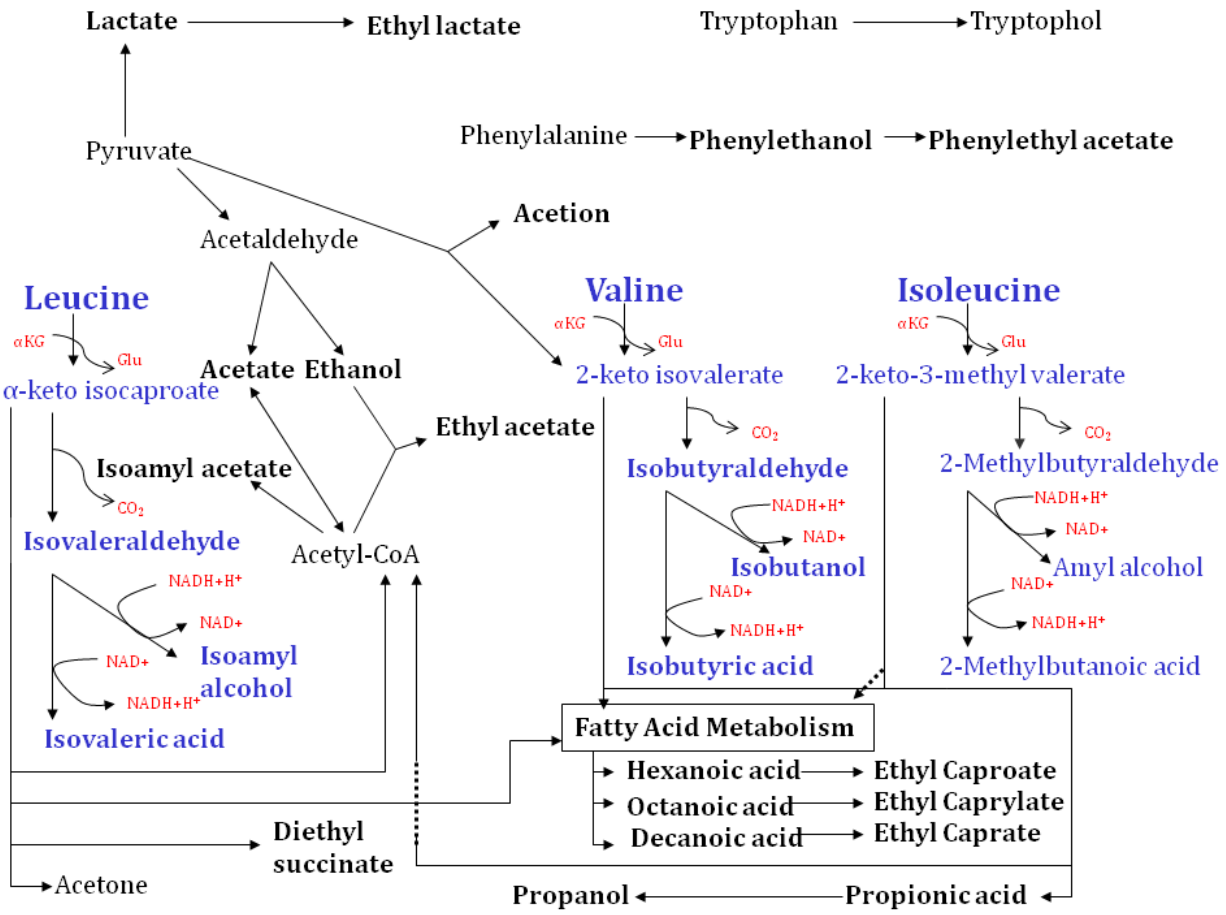



**Fig. 5.1.** A simplified metabolic map of yeast aroma compound production, indicating known metabolic linkages. Bold type indicates aroma compounds important to this study. Compounds shown in blue colour constitute a diagrammatical representation of the Ehrlich pathway, responsible for the production of higher alcohols and volatile acids. Co-factors and transition metabolites are shown in red. αKG is α-keto glutamate and Glu is glutamate.

As shown in **Figure 5.1** [38], the first step of the Ehrlich pathway involves a trans- or de-amination reaction where the amino group of the amino acid is transferred to α-ketoglutarate to form an α-keto acid and glutamate [39-41] . Thus α-keto isocaproic acid is formed from leucine, α-keto isovaleric acid from valine, and α-keto-β-methyl valeric acid from isoleucine [42-45]. This reaction is catalysed by branched-chain amino acid aminotransferases encoded by the *BAT1* and *BAT2* genes [46-51].

The second step in the Ehrlich pathway is the decarboxylation of the α-keto acid into an aldehyde [52]. Thus, isovaleraldehyde is formed from α-keto isocaproic acid, isobutyraldehyde

from α-keto isovaleric acid and 2-methyl butyraldehyde is formed from α-keto-β-methyl valeric acid [42-45, 53, 54]. The final fate of the branched-chain amino acid is thought to depend in part on the redox status of the yeast cell, as the pathway splits in two [55]. The aldehyde can either be reduced via a NADH-dependent reaction to its respective higher alcohol, i.e. isoamyl alcohol is formed from isovaleraldehyde, isobutanol is formed from isobutyraldehyde and active amyl alcohol is formed from 2-methyl butyraldehyde [42, 43, 54]; or it can be oxidized via a $NAD^+$ dependent reaction into a volatile fatty acid. If this occurs, iso-valeric acid is formed from isovaleraldehyde, iso-butyric acid is formed from isobutyraldehyde and 2-methyl butanoic acid is formed from 2-methyl butyraldehyde [42, 44, 54].

The oxidation/reduction characteristic of the last two steps of the Ehrlich pathway has given rise to the hypothesis that higher alcohols or volatile acids are at least in part formed to help maintain the NADH/$NAD^+$ ratio and redox balance of the cell [56, 57]. Some authors, however, believe that the cell produces enough acetaldehyde to fulfil this role [58]. Other hypothesis for higher alcohol production is the removal of toxic aldehyde compounds or as an alternative source for the cell to obtain nitrogen [58, 59].

A previous screening study of the EUROSCARF deletion library yielded 10 genes that most strongly impacted on higher alcohol production and therefore likely on the Ehrlich pathway, either directly or indirectly. In this case, the deletion of these genes led to decreases in the levels of higher alcohols, volatile fatty acids and other aroma compounds. The selected genes encoded 5 dehydrogenase genes, 2 reductases and 3 decarboxylases. The dehydrogenase genes include genes involved in major cellular redox maintenance reactions, such as *GPD2* - encoding the NAD-dependent glycerol 3-phosphate dehydrogenase located in the cytosol and mitochondrion [60], responsible for the conversion of dihydroxy acetone phosphate to glycerol-3-phosphate with the simultaneous oxidation of NADH to $NAD^+$ [61, 62]; *ADH3* – encoding the mitochondrial alcohol dehydrogenase isozyme III [63] involved in the NADH-dependent

reduction of acetaldehyde to ethanol and playing a part in the maintenance of the redox balance of the mitochondrion [64, 65], as well as general redox reactions in the case of *OYE2* – encoding the well conserved NADPH oxidoreductase flavin mononucleotide [66-73]. These data clearly demonstrate the sensitivity of the Ehrlich pathway, as well as of the general aroma production network to cellular redox homeostasis. The two remaining dehydrogenases, *AAD6* – encoding a putative aryl-alcohol dehydrogenase [74-76] and *HOM2* - encoding an aspartic beta semi-aldehyde dehydrogenase which catalyzes the second step in the common pathway for methionine and threonine biosynthesis [77, 78], do not play a significant role in general redox maintenance and are therefore likely to be directly involved in the Ehrlich reaction. However, previous studies on the *AAD* genes differed from these results [79], although there is some indication that *HOM2* can directly catalyse such reactions [80].

The three decarboxylases amongst the selected genes were *PAD1* – encoding a phenylacrylic acid decarboxylase which confers resistance to cinnamic acid and other phenylacrylic acids [81], as well as decarboxylating phenolic or aromatic carboxylic acids to their corresponding vinyl derivatives [82]; *SPE1* - encoding an ornithine decarboxylase which catalyzes the first step in polyamine biosynthesis in the cytoplasm, i.e. the decarboxylation of ornithine into putrescine [83-86] and *THI3* - encoding a probable decarboxylase, which is plays a regulatory role towards enzymes involved in thiamine biosynthesis [87-89]. Thi3p has been suggested to play a role in the catabolism of amino acids to long-chain and complex alcohols [53, 79]. It has recently been shown that the enzymes that decarboxylate aromatic amino acids, i.e. *ARO10*, can play a role in the Ehrlich pathway and may also decarboxylate non-aromatic amino acids [90]. Thus the hypothesis is that while Thi3p plays a direct role in the Ehrlich pathway, the role of Pad1p and Spe1p are of a more promiscuous nature and that they accept a wide range of substrates, enabling them to decarboxylate the branched-chain amino acids in the Ehrlich pathway. The reductase gene *PRO2* encodes for a gamma-glutamyl phosphate reductase and catalyzes the second step in proline biosynthesis [93]. More specifically it is responsible for the conversion of γ-Glutamyl phosphate and NADPH into L-Glutamate-γ-semialdehyde and $NADP^+$ [94]. As such

it could also play a direct role in the Ehrlich pathway as a promiscuous enzyme able to convert the aldehyde precursor to a higher alcohol or volatile fatty acid.

In this study, we further assessed the involvement of these genes in the regulation or direct catalytic activities of the Ehrlich pathway. All of these genes could present interesting targets for future strain development. For this purpose over-expression and double and triple deletion combinations of the various genes were constructed to genetically dissect this system. The data show that the transaminase reaction of the Ehrlich pathway, catalysed by Bat2p, is the main rate limiting step in the reaction, i.e. perturbation of this first reaction dominated the double and triple deletions. However, only in the case of a Δ*bat2* deletion in combination with the decarboxylase gene *THI3* and/or the dehydrogenase genes *AAD6* and *HOM2*, could a synergistic effect on the production of higher alcohols and volatile branched-chain acids above that of the Δ*bat2* strain be observed – suggesting that these genes play a central, direct role in the Ehrlich pathway.

## 5.3 MATERIALS AND METHODS

### 5.3.1 STRAINS AND GROWTH CONDITIONS

All strains used in this study and their genotypes are listed in **Table 5.1**. The medium used through-out this study was synthetic complete dextrose (SCD) medium, with the following changes or additions: SCD5+++ contained 5 % (w/v) glucose, standard concentrations of all amino acids, except increased concentrations (150 mg/l) of each of the branched-chain amino acids, leucine, isoleucine and valine. All small-scale fermentations were performed in triplicate.

Strains were grown overnight in 5 ml of YPD medium and inoculated into the sample culture at an optical density (OD) at 600nm of 0.1. Sample cultures consisted of 80 ml medium in 200 ml and were placed on a shaking incubator at 30 ºC. The bottles were sealed with only tin-foil and no fermentation caps, resulting in growth conditions that were self-anaerobic. Weight loss was measured each day to determine the rate and stage of fermentation. After 7 days, a final measurement was taken to ensure that results could be normalized, and the cell suspension was centrifuged at 5000 rpm for 5 minutes. The cell-free supernatant was used for gas chromatographic analysis.

### 5.3.2 RECOMBINANT DNA AND PLASMID CONSTRUCTION

Standard procedures for isolation and manipulation of DNA were used throughout this study [95]. Restriction enzymes, T4 DNA ligase (Roche) and ExTaq DNA polymerase (Fermentas) were used in the enzymatic manipulation of DNA according to the specifications of the supplier.

The first set of primers listed in **Table 5.2** were used to amplify the open reading frames (ORF) of the different genes by means of the polymerase chain reaction (PCR) technique (ExTaq Polymerase, TaKaRa). To identify possible cloning artefacts, all inserts were sequenced. Genomic DNA from the wild type BY4742 yeast strain was used as template to amplify the open reading frame (ORF) of the following genes: *AAD6, ADH3*, *GPD2*, *HOM2*, *OYE2*, *PAD1, PRO2*, *QCR2, SPE1* and *THI3.* This enabled direct correlations between deletion and over-expression strains, as the same genes from the same genetic background was implicated in both. The fragments were analysed by gel electrophoresis to determine whether correct sized fragments were produced by the PCR technique, after which the fragments were subcloned into pGEM-T-easy vector (Promega).

**Table 5.1**: Microbial strains and plasmids used in this study

Microbial strains and plasmids used in this study

| Strain or plasmid | Genotype or construct | Reference or source |
|---|---|---|
| *Escherichia coli* DH5α | F' endA1 hsdR17 (rk- mk+) supE44 thi-1 recA1 gyrA (Nalr) relA1 D(laclZYA-argF)U169 deoR [F80dlac DE(lacZ)M15] | GIBCO-BRL/Life Technologies |
| *Saccharomyces cerevisiae* | | |
| Laboratory control strains | | |
| BY4742 | *MAT*α his3Δ leu2Δ lys2Δ ura3Δ | EUROSCARF |
| FY23 | *MATa ura3-52 trp1-Δ63 leu2-Δ1* | Winston, et al. 1995 [96] |
| Transformants | | |
| BY4742 based: | | |
| Δ*aad6* | *MATα his3Δ leu2Δ lys2Δ ura3Δ aad6Δ::URA3* | This Study |
| Δ*adh3* | *MATα his3Δ leu2Δ lys2Δ ura3Δ adh3Δ::URA3* | This Study |
| Δ*gpd2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ gpd2Δ::URA3* | This Study |
| Δ*hom2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ hom2Δ::URA3* | This Study |
| Δ*oye2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ oye2Δ::URA3* | This Study |
| Δ*pad1* | *MATα his3Δ leu2Δ lys2Δ ura3Δ pad1Δ::URA3* | This Study |
| Δ*pro2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ pro2Δ::URA3* | This Study |
| Δ*qcr2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ qcr2Δ::URA3* | This Study |
| Δ*spe1* | *MATα his3Δ leu2Δ lys2Δ ura3Δ spe1Δ::URA3* | This Study |
| Δ*thi3* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ::URA3* | This Study |
| Δ*bat2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ bat2Δ* | Lilly, et al. 2006 [51] |
| Δ*bat2*-Δ*aad6* | *MAT*α his3Δ leu2Δ lys2Δ ura3Δ bat2Δ aad6Δ::LEU2 | This Study |
| Δ*bat2*-Δ*pad1* | *MATα his3Δ leu2Δ lys2Δ ura3Δ bat2Δ pad1Δ::URA3* | This Study |
| Δ*bat2*-Δ*pro2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ bat2Δ pro2Δ::URA3* | This Study |
| Δ*bat2*-Δ*spe1* | *MATα his3Δ leu2Δ lys2Δ ura3Δ bat2Δ spe1Δ::URA3* | This Study |
| Δ*bat2*-Δ*thi3* | *MATα his3Δ leu2Δ lys2Δ ura3Δ bat2Δ thi3Δ::URA3* | This Study |
| Δ*hom2*-Δ*aad6* | *MATα his3Δ leu2Δ lys2Δ ura3Δ hom2Δ aad6Δ::URA3* | This Study |
| Δ*hom2*-Δ*pro2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ hom2Δ pro2Δ::URA3* | This Study |
| Δ*thi3*-Δ*aad6* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ aad6Δ::LEU2* | This Study |
| Δ*thi3*-Δ*hom2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ hom2Δ::LEU2* | This Study |
| Δ*thi3*-Δ*pad1* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ pad1Δ::LEU2* | This Study |
| Δ*thi3*-Δ*pro2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ pro2Δ::LEU2* | This Study |
| Δ*thi3*-Δ*spe1* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ spe1Δ::LEU2* | This Study |
| Δ*bat2*-Δ*thi3*-Δ*aad6* | *MATα his3Δ leu2Δ lys2Δ ura3Δ bat2Δ thi3Δ::URA3 aad6Δ::LEU2* | This Study |
| Δ*bat2*-Δ*thi3*-Δ*hom2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ bat2Δ thi3Δ::URA3 hom2Δ::LEU2* | This Study |
| Δ*hom2*-Δ*pro2*-Δ*aad6* | *MATα his3Δ leu2Δ lys2Δ ura3Δ hom2Δ pro2Δ::URA3 aad6Δ::LEU2* | This Study |
| Δ*thi3*-Δ*hom2*-Δ*aad6* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ hom2Δ::LEU2 aad6Δ::LEU2* | This Study |
| Δ*thi3*-Δ*spe1*-Δ*pro2* | *MATα his3Δ leu2Δ lys2Δ ura3Δ thi3Δ spe1Δ::LEU2 pro2Δ::URA3* | This Study |
| FY23 based: | | |
| FY-pSTA | *MATa ura3-52 trp1*-Δ63 leu2-Δ1 URA3 PGK1p-PGK1t | This Study |
| FY-AAD6 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-AAD6-PGK1t* | This Study |
| FY-ADH3 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-ADH3-PGK1t* | This Study |

| | | |
|---|---|---|
| FY-GPD2 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-GPD2-PGK1t* | This Study |
| FY-HOM2 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-HOM2-PGK1t* | This Study |
| FY-OYE2 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-OYE2-PGK1t* | This Study |
| FY-PAD1 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-PAD1-PGK1t* | This Study |
| FY-PRO2 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-PRO2-PGK1t* | This Study |
| FY-QCR2 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-QCR2-PGK1t* | This Study |
| FY-SPE1 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-SPE1-PGK1t* | This Study |
| FY-THI3 | *MATa ura3-52 trp1-Δ63 leu2-Δ1 URA3 PGK1p-THI3-PGK1t* | This Study |
| Plasmids | | |
| pSTA1 | $Ap^R$ $Tc^R$$PGK_{PT}$ URA3 | Nivitha Ramachandran |

The ligated pGEM-T-easy vectors, containing the ORF's of interest were transformed into *Escherichia coli* DH5α cells using the heat-shock method [97]. Fragments were excised from pGEM-T-easy using the following restriction enzymes: *EcoRI* and *XhoI* for *AAD6*, *OYE2*, *PAD1*, *PRO2*, *QCR2* and *SPE1*; *HindIII* and *XhoI* for *ADH3*, *GPD1* and *THI3* and *SrfI* and *XhoI* for *HOM2*. These fragments were cloned into the corresponding sites on the pSTA1 vector, leading to the creation of over-expression cassettes with the ORF preceded by the phosphoglycerate kinase (PGK) promoter sequence and followed by the PGK terminator sequence. The integration plasmids were linearised with the unique *Apa*I site within the *URA*3 sequence to ensure targeting of the *URA3* locus in the FY23 *S. cerevisiae* genome. The reference strain was constructed by transforming the linearised native pSTA1 plasmid into FY23. Transformants were selected on solid SC medium (2% agar, Difco) without uracil.

Deletion strains were generated by using the second set of primers listed in **Table 5.2**. The yeast disruption plasmid, YDp-U, containing the *URA*3 gene was used as template for PCR with primers designed to amplify the *URA*3 gene, with 50 base pair (bp) overhangs, specific to either the 5' or 3' flanking sequences of the ten genes, i.e. *AAD6, ADH3*, *GPD2*, *HOM2*, *OYE2*, *PAD1,*

**Table 5.2:** Primers used in this study

| Primer name | Sequence | Enzyme |
|---|---|---|
| SET 1 | | |
| EcoRI-AAD6-F | 5'-**GAATTC**ATGGCTGATTTATTTGCTCC-3' | *Eco*RI |
| XhoI-AAD6-R | 5'-**CTCGAG**ACAGGTTCCATTTACCTTGA-3' | *Xho*I |
| HindIII-ADH3-F | 5'-**AAGCTT**ATGTTGAGAACGTCAACATT-3' | *Hind III* |
| XhoI-ADH3-R | 5'-**CTCGAG**TTTACTAGTATCGACGACGT-3' | *Xho*I |
| HindIII-GPD2-F | 5'-**AAGCTT**ATGCTTGCTGTCAGAAGATT-3' | *Hind III* |
| XhoI-GPD2-R | 5'-**CTCGAG**TTCGTCATCGATGTCTAGCT-3' | *Xho*I |
| SrfI-HOM2-F | 5'-**GCCCGGGC**ATGGCTGGAAAGAAAATTGC-3' | *Srf*I |
| XhoI-HOM2-R | 5'-**CTCGAG**AATCAAGTTTCTTGCTAGTA-3' | *Xho*I |
| EcoRI-OYE2-F | 5'-**GAATTC**ATGCCATTTGTTAAGGACTT-3' | *Eco*RI |
| XhoI-OYE2-R | 5'-**CTCGAG**ATTTTTGTCCCAACCGAGTT-3' | *Xho*I |
| EcoRI-PAD1-F | 5'-**GAATTC**ATGCTCCTATTTCCAAGAAG-3' | *Eco*RI |
| XhoI-PAD1-R | 5'-**CTCGAG**CTTGCTTTTTATTCCTTCCC-3' | *Xho*I |
| EcoRI-PRO2-F | 5'-**GAATTC**ATGTCCAGTTCACAACAAAT-3' | *Eco*RI |
| XhoI-PRO2-R | 5'-**CTCGAG**TAATGTCACAGTCTTTATATCTAA-3' | *Xho*I |
| EcoRI-QCR2-F | 5'-**GAATTC**ATGTTGTCAGCAGCTAGATT-3' | *Eco*RI |
| XhoI-QCR2-R | 5'-**CTCGAG**CAATTCGTCCAAATATGGCA-3' | *Xho*I |
| EcoRI-SPE1-F | 5'-**GAATTC**ATGTCTAGTACTCAAGTAGGAAATG-3' | *Eco*RI |
| XhoI-SPE1-R | 5'-**CTCGAG**ATCGAGTTCAGAGTCTATGTA-3' | *Xho*I |
| HindIII-THI3-F | 5'-**AAGCTT**ATGAATTCTAGCTATACACAGA-3' | *Hind III* |
| XhoI-THI3-R | 5'-**CTCGAG**GTATCCAACTTGATTTTTTT-3' | *Xho*I |
| SET 2 | | |
| YDp-AAD6-F | 5'-ATGGCTGATTTATTTGCTCCTGCTCCTGAACCATCTACAGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-AAD6-R | 5'-TTCAACAGGTTCCATTTACCTTGATAGATGCTAAAAGGGGGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-ADH3-F | 5'-ATGTTGAGAACGTCAACATTGTTCACCAGGCGTGTCCAACGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-ADH3-R | 5'-TATTATTTACTAGTATCGACGACGTATCTACCCAAAATCTGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-GPD2-F | 5'-ATGCTTGCTGTCAGAAGATTAACAAGATACACATTCCTTAGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-GPD2-R | 5'-TATTCGTCATCGATGTCTAGCTCTTCAATCATCTCCGGTAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-HOM2-F | 5'-ATGGCTGGAAAGAAAATTGCTGGTGTTTTGGGTGCTACTGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-HOM2-R | 5'-CCTTAAATCAAGTTTCTTGCTAGTAAGATTTCGGCAATCAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-OYE2-F | 5'-ATGCCATTTGTTAAGGACTTTAAGCCACAAGCTTTGGGTGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-OYE2-R | 5'-ATTTTTGTCCCAACCGAGTTTTGAGCTTCTTCGTACGTAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-PAD1-F | 5'-ATGCTCCTATTTCCAAGAAGAACTAATATAGCCTTTTTCAGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-PAD1-R | 5'-GTTACTTGCTTTTTATTCCTTCCCAACGAGGAAAAGTGTCGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-PRO2-F | 5'-ATGTCCAGTTCACAACAAATAGCCAAAAATGCCCGTAAAGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-PRO2-R | 5'-TATAATGTCACAGTCTTTATATCTAAATCCTTGTGAACAAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-QCR2-F | 5'-ATGTTGTCAGCAGCTAGATTGCAATTTGCCCAGGGGTCAGGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-QCR2-R | 5'-CTCTTACAATTCGTCCAAATATGGCAAGTTGGAAACATCAGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-SPE1-F | 5'-ATGTCTAGTACTCAAGTAGGAAATGCTCTATCTAGTTCCAGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-SPE1-R | 5'-CAATCGAGTTCAGAGTCTATGTATACTATATCCGCAGTCTGCTGCAGGTCGACGGATCCG-3' | - |
| YDp-THI3-F | 5'-ATGAATTCTAGCTATACACAGAGATATGCACTGCCGAAGTGAATTCCCGGGGATCCGGTG-3' | - |
| YDp-THI3-R | 5'-TCAGTATCCAACTTGATTTTTTTTTAGAAGTGGTTGGAATGCTGCAGGTCGACGGATCCG-3' | - |

* The enzyme sites are indicated in boldface and the region homologous to the corresponding genes are underlined

*PRO2*, *QCR2, SPE1* and *THI3*. Resulting disruption cassettes were transformed into BY4742 yeast cells, and successful transformants would insert a functional *URA*3 gene into the target gene. Transformants were selected on SC solid medium (2% agar, Difco) without uracil. For subsequent double and triple deletion strains, the yeast disruption plasmid YDp-L, containing the *LEU2* gene was used as template for PCR with primers designed to amplify the *LEU2* gene, with 50 bp overhangs, specific to either the 5' or 3' flanking sequences of one of the ten target genes, i.e. *AAD6, ADH3*, *GPD2*, *HOM2*, *OYE2*, *PAD1, PRO2*, *QCR2, SPE1* or *THI3*. Resulting disruption cassettes were transformed into single or double deletion strains, and successful transformants would insert a functional *LEU2* gene into the target gene. Transformants were selected on SC solid medium (2% agar, Difco) without uracil and leucine. Genomic DNA was isolated from both deletion and over-expression strains and PCR based confirmation of the transformations was validated with various sets of primers.

### 5.3.3 GAS-CHROMATOGRAPHIC ANALYSIS

Extraction of volatile compounds from the cell-free supernatant was done by liquid-liquid extraction. To each sample of 5 ml cell-free supernatant, 0.1 µl of an internal standard (4-methyl-2-pentanol, 500 mg/l in 12% (v/v) ethanol) was added. The mixture was extracted with 1 ml of diethyl ether for 5 minutes in an ultrasonic bath, followed by centrifugation at 4000 rpm for 3 minutes. The diethyl ether layer was removed and dried on anhydrous sodium sulphate ($Na_2SO_4$) before being injected into the gas chromatograph.

The analysis of volatile compounds was carried out on a Hewlett Packard 6890 gas chromatograph equipped with a split-splitless injector and a flame ionisation detector (FID). A DB-FFAP capillary GC column (Agilent Technologies, Little Falls, Wilmington, USA) with dimensions 60 m length $\times$ 0.25 mm internal diameter with a 0.5 $\mu$m film thickness, was used for separation. Hydrogen was used as the carrier gas at a constant flow rate of 3.3 ml/min. The injector temperature was 200°C, the split ratio 15:1, the split flow rate 49.5 ml/min, and injection

volume of 3 μl. The FID was operated at 250 ºC. The oven temperature program was as follows: 33°C (17 min) to 240°C (5 min) at 12°C/min. For each of the compounds analyzed, an internal calibration curve was constructed using known amounts of authentic standards. The internal standard and the chemicals were sourced from Merck (Cape Town, South Africa).

### 5.3.4 MODELING OF METABOLIC INTERACTIONS

The software program GenMapp was used to modify and adapt the already existing map of isoleucine, leucine and valine degradation from the Kyoto Encyclopaedia of Genes and Genomes (KEGG). Although the main function of this program is to overlay microarray data onto metabolic pathways, it is also possible to design novel, or specialist gene databases. The data obtained from the GC analysis of the different deletion strains were treated as a pseudo-microarray. The ratio of deletion strain sample to the wild type sample was calculated and transformed to log2 in order to represent up- and downward trends equally. Microarray visualisation software from The Institute for Genomic Research (TIGR), TMev, was used to visualise the GC data and to cluster deletion strains with regards to their metabolic profile. A gene database where the aroma compounds were treated as genes was compiled and linked to the metabolic map. Lastly the transformed GC data was fitted onto the map and any possible causal interactions were investigated.

### 5.3.5 STATISTICAL ANALYSIS

The statistical differences between the various deletion and over-expression strains and the wild type reference strains on the GC data were determined using one-way ANOVA, followed by the Holm-Sidak post-test to determine statistical variability between the different strains, and where a $\rho$ value of $\leq 0.01$ would mean that the differences were significant.

## 5.4 RESULTS AND DISCUSSION

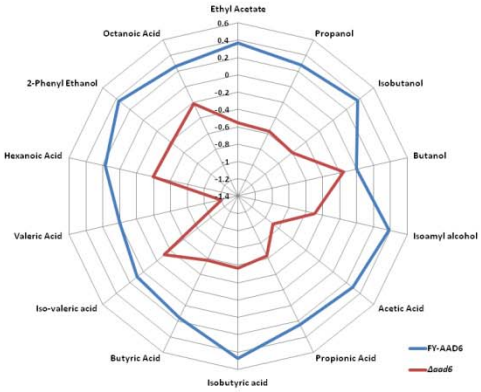
### 5.4.1 OVER-EXPRESSION *VS*. DELETION OF GENES PREVIOUSLY IDENTIFIED AS IMPACTING ON HIGHER ALCOHOL PRODUCTION

Previous work (Styger *et al*., this dissertation Chapter 4) identified 10 genes whose deletion led to notable decreases in the concentrations of the higher alcohols isoamyl alcohol and isobutanol as well as their related volatile acids, i.e. iso-valeric and iso-butyric acid. The deletion of these genes also had an impact on other unrelated aroma compounds, thus influencing the complete aroma profile of the particular strain. To further confirm the impact of these genes, independent deletions, as well as over-expression constructs using the constitutive promoter of the phoshoglycerate kinase (PGK) were also made for all selected genes. Independent over-expression of the genes should be able to eliminate other secondary impacts observed in deletion strains such as the neighbouring gene effect. Due to several reports and findings in our own laboratory suggesting that a significant number of phenotypes of EUROSCARF deletion strains may not be directly due to the deleted gene, but be due to secondary mutations in those strains we independently deleted the 10 selected genes and re-assessed their impact on the metabolism of higher alcohols and related compounds. The resulting comparisons between the EUROSCARF strains and independently deleted strains showed that in all cases except *QCR2* there was a good correlation between the EUROSCARF and independently deleted strains.

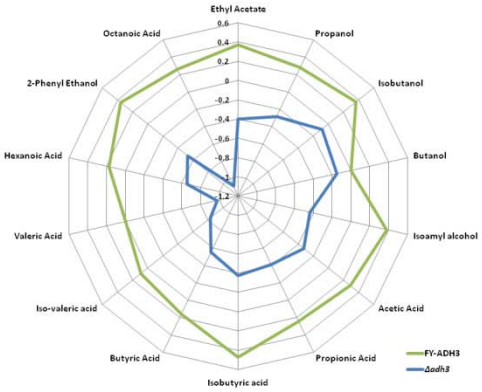
The aroma profiles produced by the deletion and over-expression strains in fermentation conditions were plotted against one another as a log2 ratio of the strain against Wild Type in **Figure 5.2**. The radar plots in the figure indicates the changes of the aroma profiles due to gene perturbation as a log 2 ratio of the perturbed strain against the consensus wild type strain. In the case of the deletion strains this is BY4742 and for the over-expression strains, the control is the isogenic FY23. These two strains are isogenic, and the FY23 strain was used to construct the over-expression strains due to difficulties in implementing our transformation strategy with

BY4742. Another approach could have been the linearization of the plasmid inside the target gene, but it was decided to minimise any perturbations of the target genes in order to prevent any artefacts forming during the transformation process. In addition the aroma profiles of the two wild type strains were very similar, but to enable direct comparisons between the deletion and over-expression strains, a consensus wild type was calculated and all deletion and over-expression strains were normalised to this consensus wild type. The complete dataset can be seen in **Table 5.3**.

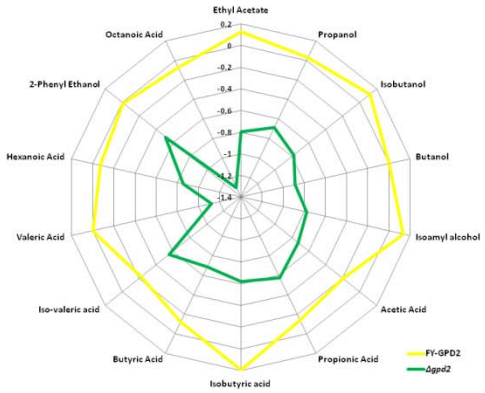
None of the genes over-expressed in the strains listed in **Table 5.3** had any notable effect on the concentrations of isobutanol and isoamyl alcohol, with the exception of the *OYE2* over-expression strain. Over-expression of *SPE1* and *THI3* led to increases in the concentration of iso-butyric acid. Over-expression of *HOM2* and *SPE1* also led to, albeit smaller, increases in the production of iso-valeric acid. These results can be clearly seen in the radar plots shown in **Figure 5.2**. An important observation from the radar plots is that, with a few exceptions, the plots for the deletion strains separate well from those of the over-expression. This means that in most cases the effect of a deletion of a certain gene on the production of a certain metabolite is reversed when the gene is over-expressed. The fact that the over-expression of these genes does not have such a significant impact on the aroma profile as the deletion of the same gene is not surprising, since availability of precursors may limit the possibilities for further increases.


Ethyl Acetate
Propanol
Isobutanol
Butanol
Isoamyl alcohol
Acetic Acid
Propionic Acid
Isobutyric acid
Butyric Acid
Iso-valeric acid
Valeric Acid
Hexanoic Acid
2-Phenyl Ethanol
Octanoic Acid
FY-AAD6
Δaad6

A


Ethyl Acetate
Propanol
Isobutanol
Butanol
Isoamyl alcohol
Acetic Acid
Propionic Acid
Isobutyric acid
Butyric Acid
Iso-valeric acid
Valeric Acid
Hexanoic Acid
2-Phenyl Ethanol
Octanoic Acid
FY-ADH3
Δadh3

B


Ethyl Acetate
Propanol
Isobutanol
Butanol
Isoamyl alcohol
Acetic Acid
Propionic Acid
Isobutyric acid
Butyric Acid
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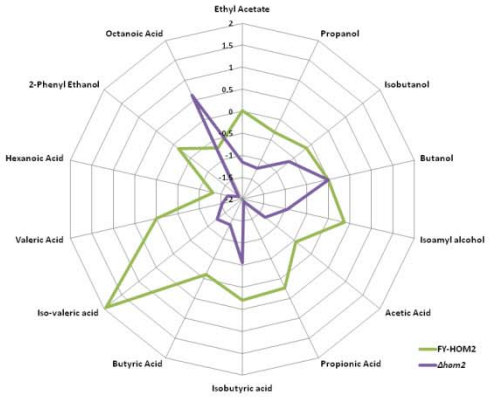
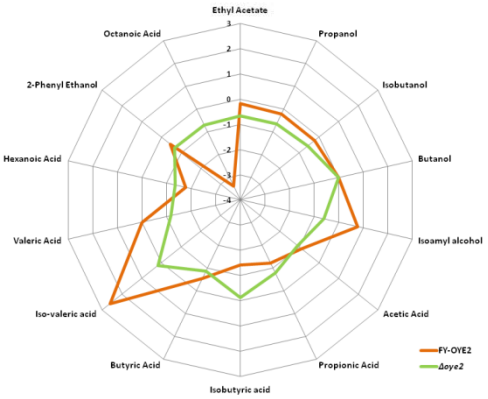
Valeric Acid
Hexanoic Acid
2-Phenyl Ethanol
Octanoic Acid
FY-GPD2
Δgpd2

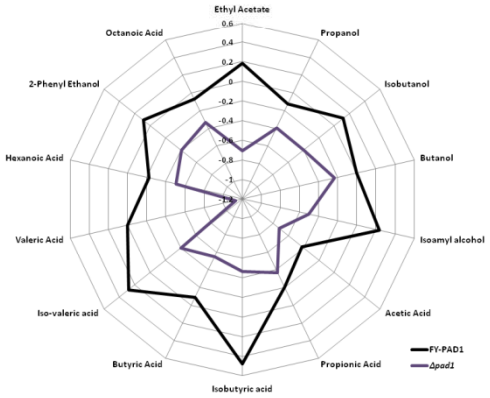
C


Ethyl Acetate
Propanol
Isobutanol
Butanol
Isoamyl alcohol
Acetic Acid
Propionic Acid
Isobutyric acid
Butyric Acid
Iso-valeric acid
Valeric Acid
Hexanoic Acid
2-Phenyl Ethanol
Octanoic Acid
FY-HOM2
Δhom2

D


Ethyl Acetate
Propanol
Isobutanol
Butanol
Isoamyl alcohol
Acetic Acid
Propionic Acid
Isobutyric acid
Butyric Acid
Iso-valeric acid
Valeric Acid
Hexanoic Acid
2-Phenyl Ethanol
Octanoic Acid
FY-OYE2
Δoye2

E


Ethyl Acetate
Propanol
Isobutanol
Butanol
Isoamyl alcohol
Acetic Acid
Propionic Acid
Isobutyric acid
Butyric Acid
Iso-valeric acid
Valeric Acid
Hexanoic Acid
2-Phenyl Ethanol
Octanoic Acid
FY-PAD1
Δpad1

F

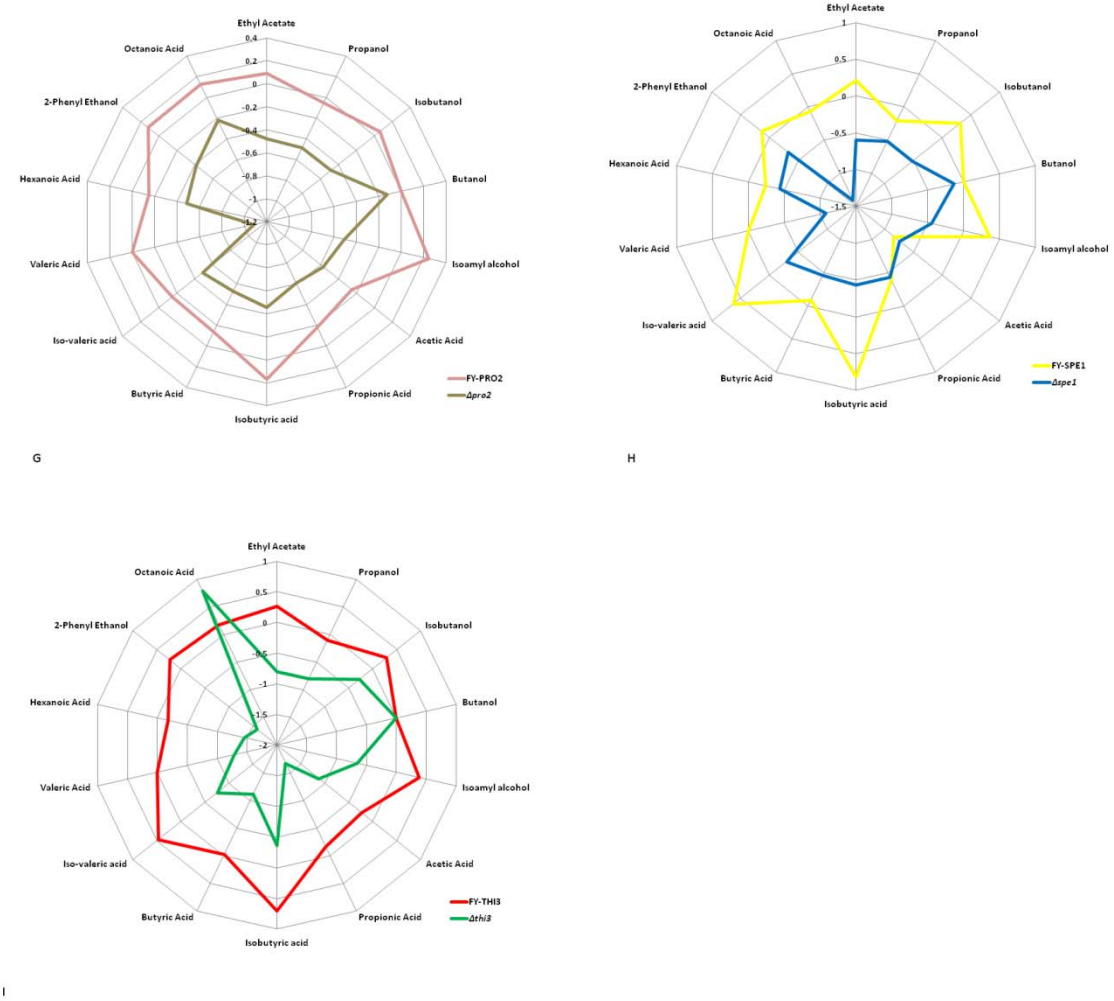



**Fig. 5.2.** Radar plots of the effect of deletion *vs*. over-expression of genes previously shown to have an impact on the Ehrlich pathway and on the yeast aroma profile; *AAD6* (A), *ADH3* (B) *GPD2* (C), *HOM2* (D), *OYE2* (E), *PAD1* (F), *PRO2* (G), , *SPE1* (H) and *THI3* (I). Values plotted are the log2 ratios of deletion strain *vs*. WT (BY4742) and over-expression strain *vs*. FY23.

**Table 5.3**: Gas Chromatographic (GC) data for single deletion strains and over-expression strains grown for one week in SCD5+++ medium. The results are the average of three replicates ± the standard deviation

| | Ethyl Acetate | Propanol | Isobutanol | Butanol | Isoamyl alcohol | Acetic Acid | Propionic Acid | Isobutyric acid | Butyric Acid | Iso-valeric acid | Valeric Acid | Hexanoic Acid | 2-Phenyl Ethanol | Octanoic Acid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus WT | 12.29 ± 1.16 | 29.23 ± 2.11 | 71.04 ± 4.38 | 0.86 ± 0.03 | 61.95 ± 1.45 | 1227.07 ± 252.90 | 8.08 ± 1.04 | 8.07 ± 0.53 | 1.83 ± 0.18 | 1.96 ± 0.21 | 0.93 ± 0.33 | 1.32 ± 0.20 | 70.90 ± 5.49 | 0.73 ± 0.40 |
| Δaad6 | 8.36 ± 0.40 | 19.63 ± 0.91 | 46.89 ± 2.22 | 0.78 ± 0.04 | 43.84 ± 2.42 | 663.73 ± 42.27 | 5.20 ± 0.34 | 5.45 ± 0.31 | 1.22 ± 0.08 | 1.58 ± 0.10 | 0.40 ± 0.02 | 1.00 ± 0.08 | 52.92 ± 4.13 | 0.63 ± 0.19 |
| Δadh3 | 9.33 ± 0.35 | 24.01 ± 1.46 | 66.85 ± 3.20 | 0.78 ± 0.03 | 45.84 ± 1.78 | 976.07 ± 67.25 | 6.06 ± 0.57 | 6.23 ± 0.22 | 1.25 ± 0.05 | 1.10 ± 0.04 | 0.47 ± 0.03 | 0.84 ± 0.04 | 48.98 ± 1.96 | 0.35 ± 0.11 |
| Δgpd2 | 7.09 ± 0.10 | 18.15 ± 0.32 | 41.43 ± 0.90 | 0.47 ± 0.36 | 36.17 ± 0.59 | 743.10 ± 81.95 | 5.42 ± 0.43 | 5.24 ± 0.21 | 1.13 ± 0.04 | 1.34 ± 0.06 | 0.43 ± 0.02 | 0.73 ± 0.04 | 49.71 ± 2.65 | 0.30 ± 0.13 |
| Δhom2 | 5.49 ± 0.05 | 12.62 ± 0.11 | 45.76 ± 0.34 | 0.00 ± 0.00 | 32.03 ± 0.15 | 483.46 ± 4.12 | 2.10 ± 0.06 | 5.46 ± 0.06 | 0.71 ± 0.01 | 0.81 ± 0.01 | 0.32 ± 0.00 | 0.42 ± 0.01 | 18.95 ± 0.15 | 1.12 ± 0.02 |
| Δoye2 | 7.70 ± 0.34 | 18.61 ± 1.38 | 47.96 ± 5.28 | 0.00 ± 0.00 | 40.77 ± 1.85 | 579.47 ± 78.42 | 4.69 ± 0.72 | 7.39 ± 0.54 | 1.00 ± 0.01 | 2.20 ± 0.04 | 0.41 ± 0.02 | 0.52 ± 0.03 | 44.33 ± 0.65 | 0.45 ± 0.07 |
| Δpad1 | 7.53 ± 0.85 | 22.23 ± 4.34 | 53.96 ± 10.39 | 0.73 ± 0.07 | 43.74 ± 3.68 | 745.52 ± 107.49 | 6.25 ± 1.08 | 5.85 ± 0.82 | 1.25 ± 0.09 | 1.49 ± 0.16 | 0.42 ± 0.02 | 0.93 ± 0.12 | 53.56 ± 6.93 | 0.58 ± 0.55 |
| Δpro2 | 8.81 ± 0.58 | 20.85 ± 0.26 | 50.78 ± 0.48 | 0.79 ± 0.01 | 43.63 ± 1.32 | 824.73 ± 29.98 | 5.31 ± 0.17 | 5.89 ± 0.08 | 1.27 ± 0.02 | 1.40 ± 0.03 | 0.44 ± 0.01 | 0.95 ± 0.04 | 53.10 ± 0.80 | 0.63 ± 0.19 |
| Δqcr2 | 7.35 ± 0.61 | 21.26 ± 1.26 | 92.48 ± 8.70 | 0.72 ± 0.02 | 65.59 ± 0.33 | 536.33 ± 72.82 | 7.84 ± 1.01 | 10.78 ± 1.12 | 1.37 ± 0.06 | 2.44 ± 0.10 | 0.55 ± 0.01 | 0.62 ± 0.11 | 98.23 ± 6.40 | 0.00 ± 0.00 |
| Δspe1 | 8.10 ± 0.21 | 20.45 ± 0.85 | 49.55 ± 1.92 | 0.79 ± 0.03 | 45.41 ± 1.57 | 735.63 ± 58.02 | 5.98 ± 0.51 | 5.98 ± 0.30 | 1.32 ± 0.06 | 1.60 ± 0.06 | 0.44 ± 0.02 | 0.98 ± 0.03 | 56.56 ± 2.45 | 0.28 ± 0.04 |
| Δthi3 | 7.07 ± 0.42 | 16.84 ± 0.93 | 58.53 ± 2.39 | 0.00 ± 0.00 | 39.32 ± 1.33 | 563.42 ± 26.99 | 2.53 ± 0.13 | 6.27 ± 0.27 | 0.85 ± 0.01 | 1.16 ± 0.15 | 0.38 ± 0.02 | 0.48 ± 0.02 | 23.57 ± 1.86 | 1.27 ± 0.20 |
| FY-AAD6 | 15.88 ± 0.44 | 35.51 ± 3.24 | 91.53 ± 7.12 | 0.00 ± 0.00 | 80.68 ± 2.60 | 1501.45 ± 287.73 | 9.56 ± 1.59 | 11.22 ± 0.61 | 2.04 ± 0.15 | 2.09 ± 0.08 | 0.00 ± 0.00 | 1.49 ± 0.04 | 90.88 ± 3.27 | 0.88 ± 0.07 |
| FY-ADH3 | 14.80 ± 0.16 | 31.85 ± 2.12 | 82.03 ± 1.48 | 0.00 ± 0.00 | 70.19 ± 1.81 | 1147.72 ± 97.42 | 8.00 ± 0.51 | 9.59 ± 0.40 | 1.78 ± 0.06 | 1.87 ± 0.11 | 0.00 ± 0.00 | 1.35 ± 0.10 | 80.21 ± 3.24 | 0.90 ± 0.03 |
| FY-GPD2 | 13.41 ± 0.40 | 29.82 ± 0.49 | 77.48 ± 1.61 | 0.00 ± 0.00 | 67.93 ± 1.06 | 1070.02 ± 91.74 | 7.34 ± 0.19 | 9.22 ± 0.36 | 1.68 ± 0.03 | 1.68 ± 0.04 | 0.00 ± 0.00 | 1.26 ± 0.04 | 70.32 ± 0.71 | 0.70 ± 0.06 |
| FY-HOM2 | 12.37 ± 0.22 | 23.54 ± 0.51 | 64.57 ± 2.61 | 0.00 ± 0.00 | 80.24 ± 3.14 | 896.83 ± 94.51 | 9.48 ± 0.35 | 9.91 ± 0.80 | 1.69 ± 0.03 | 7.59 ± 0.27 | 0.00 ± 0.00 | 0.53 ± 0.04 | 64.07 ± 3.79 | 0.45 ± 0.02 |
| FY-OYE2 | 10.82 ± 0.34 | 25.18 ± 2.90 | 60.58 ± 4.28 | 0.00 ± 0.00 | 106.81 ± 1.52 | 656.86 ± 125.62 | 3.47 ± 0.19 | 2.99 ± 0.26 | 1.25 ± 0.02 | 11.96 ± 0.67 | 0.00 ± 0.00 | 0.39 ± 0.04 | 51.48 ± 1.75 | 0.07 ± 0.01 |
| FY-PAD1 | 13.98 ± 1.13 | 26.80 ± 4.42 | 77.12 ± 4.50 | 0.00 ± 0.00 | 72.88 ± 3.08 | 918.92 ± 59.54 | 7.01 ± 0.60 | 11.24 ± 1.16 | 1.72 ± 0.11 | 2.38 ± 0.30 | 0.00 ± 0.00 | 1.13 ± 0.15 | 75.58 ± 5.39 | 0.70 ± 0.11 |
| FY-PRO2 | 13.10 ± 0.58 | 28.24 ± 2.15 | 74.06 ± 5.70 | 0.00 ± 0.00 | 73.44 ± 1.53 | 1029.34 ± 54.85 | 7.13 ± 0.57 | 9.07 ± 0.59 | 1.66 ± 0.04 | 1.77 ± 0.04 | 0.00 ± 0.00 | 1.19 ± 0.05 | 77.05 ± 5.08 | 0.80 ± 0.04 |
| FY-QCR2 | 11.74 ± 0.04 | 20.82 ± 0.02 | 63.02 ± 0.38 | 0.00 ± 0.00 | 62.13 ± 0.42 | 1376.61 ± 30.65 | 6.11 ± 0.11 | 6.05 ± 0.18 | 1.37 ± 0.04 | 1.46 ± 0.05 | 0.00 ± 0.00 | 1.12 ± 0.08 | 67.11 ± 2.29 | 0.66 ± 0.02 |
| FY-SPE1 | 14.21 ± 0.42 | 25.27 ± 0.27 | 88.38 ± 3.70 | 0.00 ± 0.00 | 79.76 ± 2.73 | 685.84 ± 159.50 | 6.19 ± 0.43 | 14.22 ± 0.38 | 1.72 ± 0.02 | 3.03 ± 0.09 | 0.00 ± 0.00 | 1.12 ± 0.02 | 78.00 ± 1.98 | 0.70 ± 0.03 |
| FY-THI3 | 14.78 ± 0.66 | 27.26 ± 1.60 | 86.49 ± 7.68 | 0.00 ± 0.00 | 80.56 ± 2.42 | 1042.09 ± 121.59 | 7.25 ± 0.45 | 13.18 ± 0.58 | 1.80 ± 0.01 | 2.73 ± 0.05 | 0.00 ± 0.00 | 1.17 ± 0.09 | 83.56 ± 1.63 | 0.83 ± 0.05 |

**Table 5.4**. Gas Chromatographic (GC) data for double and triple single deletion strains grown for one week in SCD5+++ medium. The results are the average of three replicates ± the standard deviation

| | Ethyl Acetate | Propanol | Isobutanol | Butanol | Isoamyl alcohol | Acetic Acid | Propionic Acid | Isobutyric acid | Butyric Acid | Iso-valeric acid | Valeric Acid | Hexanoic Acid | 2-Phenyl Ethanol | Octanoic Acid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus WT | 12.29 ± 1.16 | 29.23 ± 2.11 | 71.04 ± 4.38 | 0.86 ± 0.03 | 61.95 ± 1.45 | 1227.07 ± 252.90 | 8.08 ± 1.04 | 8.07 ± 0.53 | 1.83 ± 0.18 | 1.96 ± 0.21 | 0.93 ± 0.33 | 1.32 ± 0.20 | 70.90 ± 5.49 | 0.73 ± 0.40 |
| Δbat2-Δaad6 | 8.79 ± 0.35 | 25.27 ± 2.23 | 27.84 ± 1.46 | 0.80 ± 0.03 | 48.04 ± 1.27 | 608.30 ± 71.16 | 6.72 ± 0.45 | 3.09 ± 0.03 | 1.24 ± 0.04 | 1.23 ± 0.03 | 0.31 ± 0.18 | 0.86 ± 0.04 | 53.18 ± 5.06 | 0.31 ± 0.05 |
| Δbat2-Δpad1 | 8.01 ± 0.83 | 19.21 ± 1.82 | 34.10 ± 1.74 | 0.68 ± 0.01 | 29.14 ± 0.27 | 743.65 ± 86.87 | 5.32 ± 0.30 | 3.70 ± 0.04 | 1.06 ± 0.02 | 0.95 ± 0.02 | 0.35 ± 0.02 | 0.67 ± 0.03 | 48.69 ± 1.07 | 0.25 ± 0.06 |
| Δbat2-Δpro2 | 10.78 ± 2.75 | 25.42 ± 1.80 | 44.39 ± 3.86 | 0.75 ± 0.01 | 31.74 ± 0.49 | 889.68 ± 368.85 | 5.62 ± 2.19 | 3.59 ± 1.48 | 1.03 ± 0.22 | 0.86 ± 0.22 | 0.41 ± 0.02 | 0.58 ± 0.11 | 50.04 ± 2.80 | 0.26 ± 0.03 |
| Δbat2-Δspe1 | 6.92 ± 0.58 | 19.10 ± 1.48 | 34.47 ± 3.08 | 0.71 ± 0.04 | 29.53 ± 1.48 | 519.39 ± 285.55 | 4.75 ± 1.24 | 3.29 ± 0.50 | 1.02 ± 0.11 | 0.93 ± 0.07 | 0.31 ± 0.02 | 0.63 ± 0.07 | 47.89 ± 5.85 | 0.16 ± 0.05 |
| Δbat2-Δthi3 | 8.63 ± 0.80 | 26.69 ± 8.08 | 48.81 ± 12.75 | 0.76 ± 0.10 | 37.26 ± 4.10 | 741.06 ± 84.30 | 7.67 ± 1.85 | 5.67 ± 1.13 | 1.35 ± 0.19 | 1.47 ± 0.19 | 0.42 ± 0.09 | 0.88 ± 0.08 | 68.16 ± 6.77 | 0.21 ± 0.04 |
| Δhom2-Δaad6 | 7.54 ± 0.34 | 20.53 ± 1.03 | 20.94 ± 0.63 | 0.58 ± 0.01 | 35.06 ± 1.37 | 635.60 ± 30.72 | 3.57 ± 0.12 | 3.53 ± 0.09 | 0.86 ± 0.02 | 2.31 ± 0.12 | 0.27 ± 0.01 | 0.50 ± 0.03 | 38.76 ± 0.93 | 0.29 ± 0.05 |
| Δhom2-Δpro2 | 9.60 ± 0.51 | 22.06 ± 2.51 | 44.64 ± 1.44 | 0.68 ± 0.01 | 46.95 ± 1.08 | 690.46 ± 75.41 | 4.14 ± 0.32 | 6.20 ± 0.31 | 0.96 ± 0.02 | 2.10 ± 0.04 | 0.26 ± 0.01 | 0.63 ± 0.02 | 34.85 ± 0.84 | 0.28 ± 0.06 |
| Δthi3-Δaad6 | 8.82 ± 0.45 | 23.95 ± 0.56 | 40.57 ± 2.34 | 0.81 ± 0.01 | 61.37 ± 2.83 | 587.38 ± 124.80 | 6.20 ± 0.22 | 4.58 ± 0.46 | 1.25 ± 0.02 | 1.51 ± 0.14 | 0.39 ± 0.01 | 0.70 ± 0.11 | 51.48 ± 4.91 | 0.07 ± 0.05 |
| Δthi3-Δhom2 | 5.78 ± 2.92 | 12.22 ± 7.59 | 39.97 ± 1.58 | 0.69 ± 0.02 | 44.59 ± 5.26 | 64.52 ± 1.10 | 1.83 ± 1.07 | 0.38 ± 0.01 | 0.71 ± 0.24 | 1.03 ± 0.01 | 0.22 ± 0.09 | 0.56 ± 0.01 | 41.00 ± 2.76 | 0.03 ± 0.01 |
| Δthi3-Δpad1 | 9.95 ± 0.35 | 27.47 ± 5.41 | 42.85 ± 4.73 | 0.84 ± 0.03 | 61.63 ± 0.76 | 758.84 ± 372.69 | 7.46 ± 1.48 | 4.91 ± 0.27 | 1.33 ± 0.07 | 1.60 ± 0.10 | 0.42 ± 0.06 | 0.86 ± 0.09 | 50.99 ± 1.84 | 0.23 ± 0.03 |
| Δthi3-Δpro2 | 8.50 ± 1.13 | 20.97 ± 1.31 | 44.44 ± 6.19 | 0.74 ± 0.07 | 43.91 ± 6.89 | 695.85 ± 115.54 | 5.52 ± 0.89 | 4.89 ± 0.76 | 1.13 ± 0.12 | 1.23 ± 0.19 | 0.36 ± 0.02 | 0.75 ± 0.12 | 43.02 ± 4.32 | 0.27 ± 0.11 |
| Δthi3-Δspe1 | 8.58 ± 1.17 | 20.49 ± 1.81 | 37.88 ± 2.23 | 0.76 ± 0.01 | 60.35 ± 5.50 | 492.36 ± 46.16 | 5.08 ± 0.30 | 5.24 ± 0.13 | 1.13 ± 0.04 | 2.38 ± 0.22 | 0.36 ± 0.02 | 0.70 ± 0.16 | 46.46 ± 3.10 | 0.26 ± 0.24 |
| Δbat2-Δthi3-Δhom2 | 5.82 ± 0.20 | 19.82 ± 0.98 | 20.25 ± 1.13 | 0.67 ± 0.03 | 31.69 ± 1.18 | 543.03 ± 52.39 | 4.69 ± 0.44 | 2.71 ± 0.14 | 0.91 ± 0.04 | 1.69 ± 0.09 | 0.36 ± 0.02 | 0.47 ± 0.02 | 38.77 ± 1.49 | 0.16 ± 0.05 |
| Δbat2-Δthi3-Δaad6 | 4.43 ± 0.13 | 16.08 ± 0.92 | 18.76 ± 1.17 | 0.58 ± 0.01 | 26.98 ± 1.02 | 389.03 ± 34.55 | 5.09 ± 0.40 | 2.20 ± 0.11 | 0.87 ± 0.03 | 0.91 ± 0.03 | 0.29 ± 0.01 | 0.43 ± 0.02 | 41.28 ± 1.48 | 0.00 ± 0.00 |
| Δhom2-Δpro2-Δaad6 | 5.97 ± 0.28 | 7.22 ± 0.23 | 37.80 ± 2.24 | 0.00 ± 0.00 | 21.20 ± 0.56 | 297.67 ± 23.67 | 1.40 ± 0.07 | 4.85 ± 0.32 | 0.59 ± 0.05 | 0.57 ± 0.11 | 0.00 ± 0.00 | 0.29 ± 0.02 | 10.50 ± 0.38 | 0.64 ± 0.05 |
| Δthi3-Δhom2-Δaad6 | 7.00 ± 0.55 | 15.26 ± 1.83 | 28.75 ± 2.01 | 0.54 ± 0.03 | 40.76 ± 3.53 | 546.33 ± 78.52 | 3.04 ± 0.24 | 4.73 ± 0.22 | 0.83 ± 0.08 | 1.45 ± 0.16 | 0.21 ± 0.01 | 0.58 ± 0.07 | 33.97 ± 1.43 | 0.52 ± 0.17 |
| Δthi3-Δspe1-Δpro2 | 5.76 ± 0.17 | 12.20 ± 0.81 | 26.09 ± 0.43 | 0.46 ± 0.01 | 31.47 ± 0.90 | 532.48 ± 48.82 | 2.74 ± 0.13 | 4.43 ± 0.21 | 0.66 ± 0.01 | 1.40 ± 0.05 | 0.19 ± 0.01 | 0.47 ± 0.02 | 28.29 ± 0.59 | 0.44 ± 0.04 |

The shape of the radar plots allows to visually group the genes according to the type of impact they exert on the aroma production network. In the case of *AAD6*, *ADH3* and *GPD2* (panels A, B and C) it is clear that the shape of the over-expression strain's radar plot shows very little variation between the three strains. The radar plots for the deletion strain also show a certain similarity in their pattern. This would indicate a similar response of the cell to over-expression or deletion of these three genes. *GPD2* and *ADH3* are major role players in cellular redox homeostasis. Their impact therefore most likely is of an indirect nature, and reflects the need of redox balance maintenance. Since both genes are major contributors to the regeneration of NAD+ under fermentative conditions, the similarity of their impact is not surprising. Therefore the data clearly show that the Ehrlich pathway is indeed sensitive to perturbations in cellular redox maintenance. *AAD6*, on the other hand, is not known to contribute significantly to redox balance maintenance. The fact that the deletion of this gene shows similar effects as those of important redox role players such as *GPD2* and *ADH3*, suggests a much more direct involvement of this gene product in the Ehrlich pathway.

In panel E, the radar plot for the gene *OYE2* is shown. It is clear that little effect is seen on the aroma profile either when the gene is deleted or over-expressed, except the 6.1 fold increase in the concentration of iso-valeric acid in the over-expression strain. The two lines representing the strains are very close together and hover around the zero mark, which in log2 terms means a fold change of one, or no effect. As discussed above, deletion or over-expression of this gene may cause perturbations in the redox homeostasis of the cell and necessitate seemingly small changes in the production of certain compounds, independently of the Ehrlich pathway.

The radar plots for the genes *HOM2*, *PAD1*, *PRO2*, *SPE1* and *THI3* (panels D, F, G, and I), however, show a much more varied picture. Here some variation in the aroma profile can be seen in both over-expression and deletion strains, hinting at more than simply a general cellular

response to a redox perturbation. *HOM2* encodes for an aspartic beta semi-aldehyde dehydrogenase, which catalyzes the second step in the common pathway for methionine and threonine biosynthesis and also plays a part in the biosynthesis of homoserine. Deletion of this gene leads to the biggest effects on the levels of higher alcohols and volatile fatty acids and because of the involvement of hom2p in amino acid metabolism it remains a important candidate gene for direct involvement in the Ehrlich pathway. In fact, *HOM2* (panel D) and *THI3* (panel I) show some similarity in their deletion and to a lesser extent in their over-expression profiles. *THI3* encodes a probable decarboxylase, thought to be involved in thiamine biosynthesis and its gene product have been shown to play a role in the catabolism of amino acids to long-chain and complex alcohols [79].

The other two decarboxylases *PAD1* and *SPE1* also have similar deletion and over-expression profiles (panels F and H). Pad1p is a phenylacrylic acid decarboxylase, which confers resistance to cinnamic acid and can also decarboxylate aromatic carboxylic acids to the corresponding vinyl derivatives in the mitochondrion. *SPE1* encodes an ornithine decarboxylase which catalyzes the first step in polyamine biosynthesis in the cytoplasm. Both these genes are involved in somewhat related biochemical reactions to those of the Ehrlich pathway – it is not inconceivable that they may also be promiscuous in nature and play a direct role in the catabolism of branched-chain amino acids. Finally, *PRO2* (panel G) encodes the gamma-glutamyl phosphate reductase, which has previously been shown to catalyze the second step in proline biosynthesis [98]. The radar plot for this gene shows good separation for deletion and over-expression strain, but with some variations in the profiles of both – once again a good indication that this gene might be involved in a more direct way than simply a general cellular response to a redox perturbation as with some of the other genes discussed.

### 5.4.2 THE EFFECT OF COMBINATION OF DOUBLE OR TRIPLE DELETION OF GENES

To further investigate the possible roles that some of the previously identified genes may play in the production of higher alcohols and related compounds, we created strains with combinations

of double, and triple deletions of selected genes that were shown to influence higher alcohol production. Firstly the effect of these combinatorial deletions on the general aroma profile will be discussed.

#### 5.4.2.1 DELETIONS IN COMBINATION WITH *BAT2*

Based on the results above it was decided to narrow the field of candidate genes for further investigation down to the six genes that appeared most likely to be directly involved in Ehrlich pathway reactions, i.e. *AAD6*, *HOM2*, *PAD1*, *PRO2*, *SPE1* and *THI3*. Combinations of double and triple deletions were created and the subsequent effect on the aroma profile was observed. The gene *BAT2* catalyzing the transamination step of the Ehrlich pathway was also included in this study. A heat map of the ratio of concentrations of various compounds from the deletion strains versus the consensus wild type expressed in log2 can be seen in **Figure 5.3** and the complete dataset of all the double and triple deletions is shown in **Table 5.4**. It is immediately clear that in almost all instances the additional deletion of one or two genes causes not only the higher alcohols and volatile fatty acids directly linked to the Ehrlich pathway to decrease notably, but that the entire aroma profile shifts away from the wild type strain with decreases seen for almost all compounds. This was not always the case for single deletion strains where the results were somewhat ambiguous.

In **Figure 5.4,** panel A, the results of double deletions with *BAT2* and the candidate genes on the yeast aroma profile are shown. All of the strains show a somewhat similar trend when investigating the aroma profiles, although the magnitude of changes for some strains are less than others. The strain that has the greatest negative impact on the overall aroma profile is the Δ*bat2*-Δ*spe1* strain. A very similar aroma profile is observed for this strain and the other transaminase-decarboxylase double gene deletion strain, Δ*bat2*-Δ*pad1*. Both of the higher alcohols, as well as the volatile fatty acids are negatively impacted upon by these double

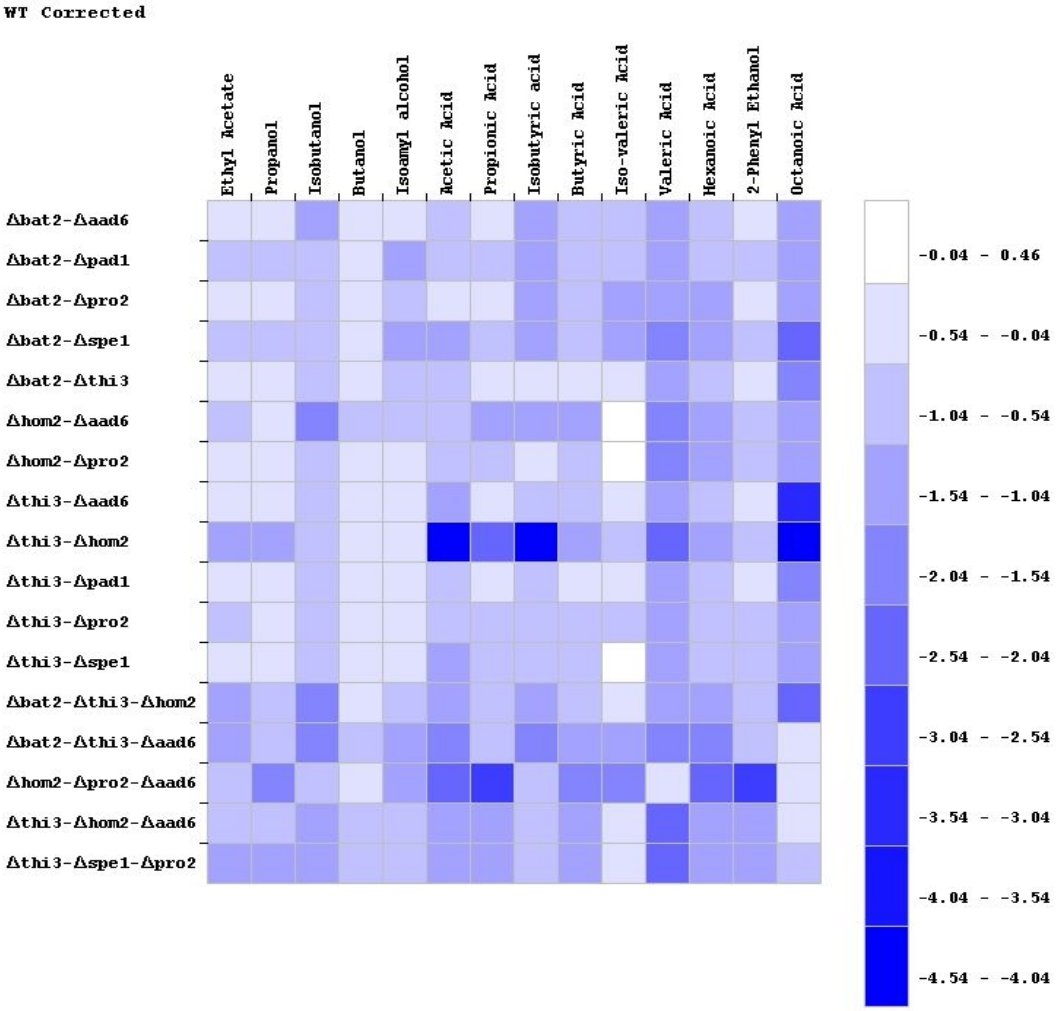



**Fig. 5.3**. Heat-map of the GC data of all the deletion strains indicating the levels of metabolites with regards to the consensus wild type strain as the log2 of their ratio. A decrease in the production of a certain metabolite is indicated by a blue square and the intensity of the colour represents the fold value as indicated by the scale on the right hand side.

deletions. Interestingly the other decarboxylase gene deletion made in combination with *BAT2*, Δ*bat2*-Δ*thi3*, differs quite substantially from the above mentioned strains. This could indicate that *THI3* plays a different – possibly more direct - role in the Ehrlich pathway than the other decarboxylase genes. The other two genes combined with *BAT2* were *AAD6*, a putative dehydrogenase and *PRO2*, a reductase. These two double deletion strains would both be perturbed in the transaminase and reduction/oxidation reactions of the Ehrlich pathway, but once again there are notable differences in the aroma profiles of the two strains. The Δ*bat2*-Δ*aad6* strain has the biggest effect on compounds related to valine catabolism, i.e. isobutanol and iso-butyric acid. This substrate specificity could indicate that although these genes can

metabolise a varied range of compounds, there might still be some differences in their specificity. Most other compounds, with the exception of valeric acid, are also negatively affected by the combined deletions.

#### 5.4.2.2 DELETIONS IN COMBINATION WITH *THI3*

The gene *THI3*, encoding a probable decarboxylase, has been shown to be involved in the regulation of enzymes involved in thiamine biosynthesis. More importantly for this study, it may also have a role in the catabolism of amino acids to long-chain and complex alcohols. In **Figure 5.4**, panel B, the effects of deletion of other genes in combination with *THI3* is shown.

The strains Δ*thi3*-Δ*aad6* and Δ*thi3*-Δ*hom2* combine a deletion of a decarboxylase with a dehydrogenase. However their aroma profiles are quite dissimilar, except for the large decrease seen in one of the down-stream metabolites of the Ehrlich pathway, i.e. octanoic acid. Furthermore, acetic acid and iso-butyric acid are notably reduced by the latter strain. The formation of both acetic- and iso-butyric acid requires oxidation i.e. the consumption of $NAD^+$. The fact that production of both these compounds are reduced to such a large extent may indicate that the deletion *HOM2*, in combination with *THI3* somehow limits the availability of $NAD^+$, leading to the inhibition of compounds requiring this co-factor.

The deletion of two decarboxylase genes, as seen in the radar plot in **Figure 5.4** depicting strains Δ*thi3*-Δ*pad1* and Δ*thi3*-Δ*spe1,* as well as the decarboxylase-reductase combination of Δ*thi3*-Δ*pro2* seem to lead to profiles that are very similar, with very little impact on the aroma profile and the biggest fold decreases seen for valeric- and octanoic acid. This would seem to indicate that the *PAD1*, *PRO2* and *SPE1* genes have very little influence on the Ehrlich pathway, when compared to genes such as *BAT2* and *THI3*.

#### 5.4.2.3 DELETIONS IN COMBINATION WITH *HOM2*

In **Figure 5.4**, panel C, the effects, measured as fold differences (in log2 values) against the wild type strain, of additional deletions in combination with a deletion of the aspartic beta semi-aldehyde dehydrogenase *HOM2*, can be seen. In the first instance, *AAD6*, a putative aryl-alcohol dehydrogenase is deleted together with *HOM2*. The second combination entails deletion of *HOM2* together with the gamma-glutamyl phosphate reductase, *PRO2*.

It is immediately clear from **Figure 5.4** that both of these strains have a similar aroma profile, Small effects such as these could however indicate fine-tuning of the Ehrlich pathway by the cell to adapt to changing conditions. The double deletion of genes both related to oxidation/reduction reactions may also place a burden on the cell with regards to redox maintenance and it may compensate in such a fashion by fine-tuning various pathways throughout the cell.

It is also clear that the effects on volatile aroma compounds are much more pronounced in the Δ*hom2*-Δ*aad6* strain, when compared with the Δ*hom2*-Δ*pro2* strain, especially when looking at the levels of isobutanol. This is also the case with iso-butyric acid, where deletion of both *HOM2* and *AAD6* causes a notable decrease against the almost no effect seen with the Δ*hom2*-Δ*pro2* strain. These observations leads to the hypothesis that of the two genes, *AAD6* and *PRO2*, it is the former that plays a bigger or more important part in the oxidation and reduction reactions of the Ehrlich pathway.

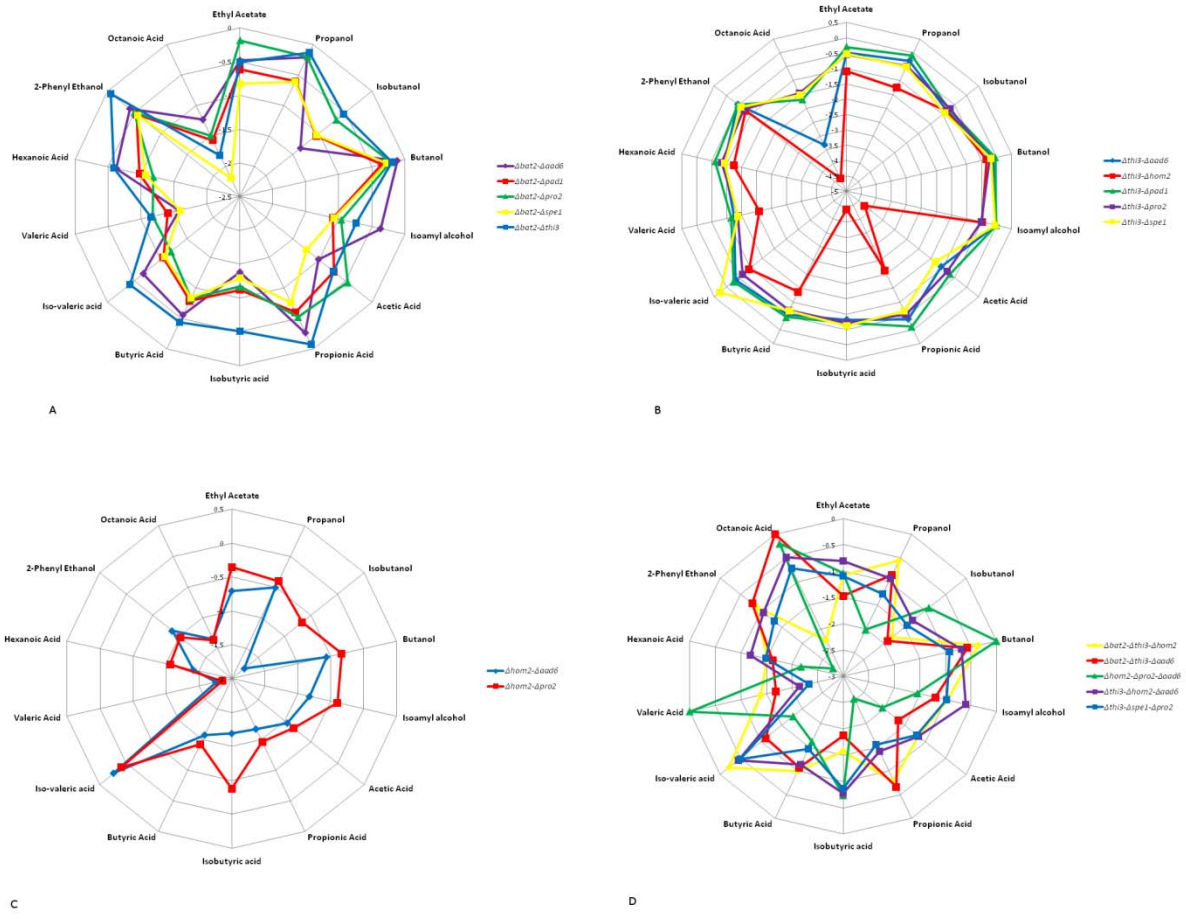



**Fig. 5.4.** Radar plots of the effect of various combinations of double and triple deletions of genes previously shown to have an impact on the Ehrlich pathway, on the yeast aroma profile; panel A represents double deletions in combination with BAT2, panel B shows double deletions in combination with *THI3,* panel C represents double deletions in combination with *HOM2* and panel D shows the effect of various combinations of triple deletions on the aroma profile. Values plotted are the log2 ratios of various deletion strains *vs*. WT (BY4742).

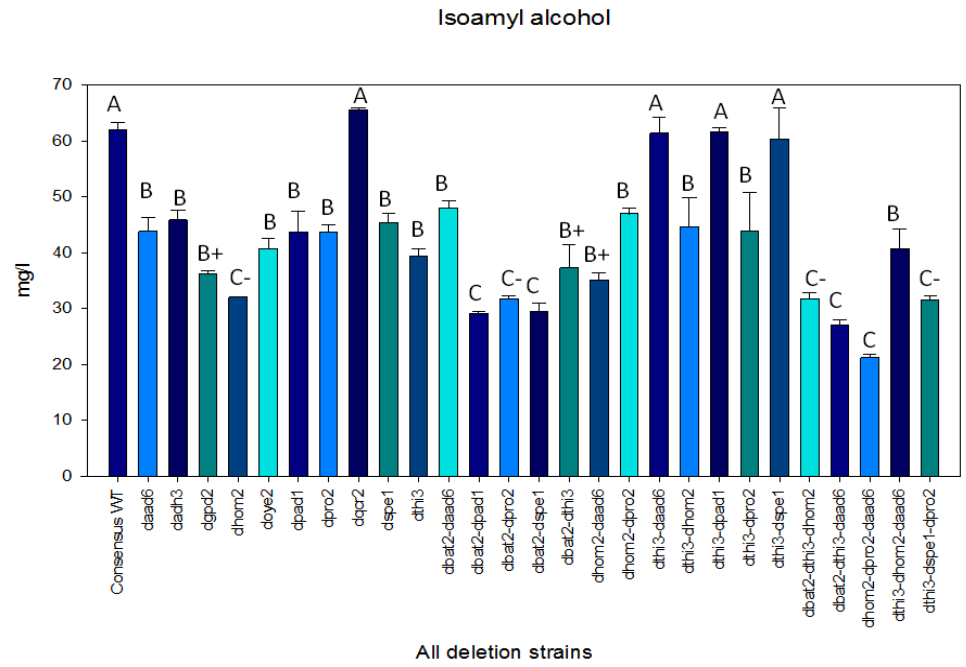
#### 5.4.2.4 TRIPLE DELETION STRAINS

In **Figure 5.4**, panel D the effect of these strains with regards to the wild type strain on the aroma profile is shown. The triple deletion strain Δ*hom2*-Δ*pro2*-Δ*aad6* represents a triple perturbation to the Ehrlich pathway in reactions by which either a higher alcohol or branched-chain acid is formed, as all three genes play a role in oxidation/reduction reactions. This strain also shows a completely individual aroma profile with regards to all the other double and triple deletion strains. Firstly no effect is seen on levels of butanol and more importantly valeric acid –

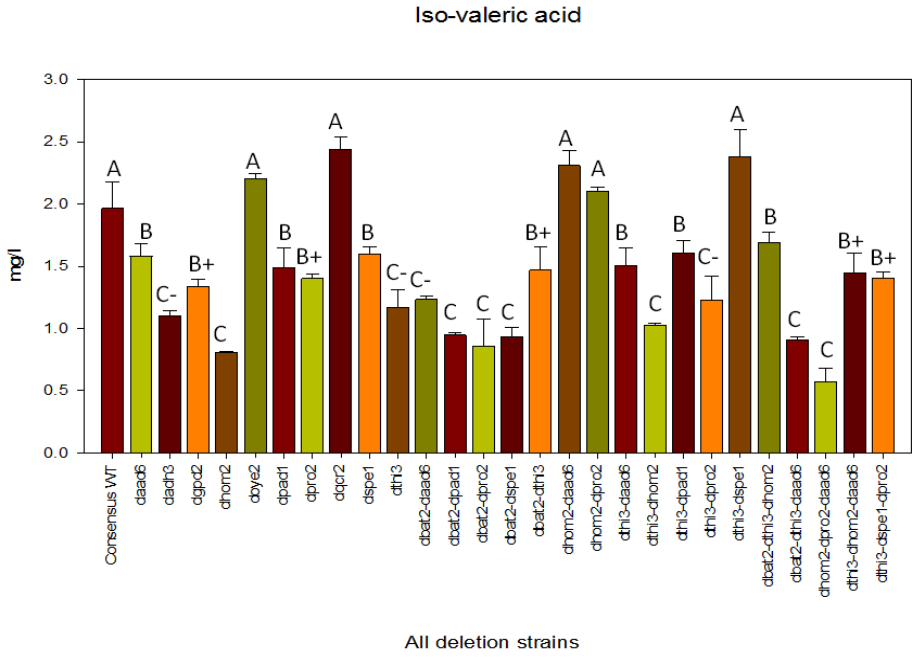
a compound that was routinely decreased when these genes were deleted. Also the higher alcohols and volatile fatty acids do not decrease notably more in this triple deletion strain than in the previously discussed double deletion strains.

However, the end concentration of the unrelated higher alcohol 2-phenyl ethanol, formed via the Ehrlich pathway from phenylalanine, decreased notably. This effect was not observed for any of the single or double deletion strains, but might once again be due to location specific redox perturbations experienced by this strain. Decreasing the production of 2-phenyl ethanol would have an effect of lessening the expenditure of NADH, a compound that might be limiting under these conditions. Another compound that shows an appreciable decline is the down-stream metabolite propionic acid.

Two triple deletion strains from **Figure 5.4** show a remarkably similar aroma profile based on the fold effects on the volatile aroma compounds produced. Both the strains have the decarboxylase *THI3* knocked-out, whilst the Δ*thi3*-Δ*hom2*-Δ*aad6* strain also has the additional double dehydrogenase deletion of *HOM2* and *AAD6*. The other strain, Δ*thi3*-Δ*spe1*-Δ*pro2*, on the other hand has a double decarboxylase deletion in *THI3* and *SPE1*, with the addition of a reductase deletion, i.e. *PRO2*. It is clear that the effects seen with the Δ*thi3*-Δ*hom2*-Δ*aad6* strain is slightly amplified when compared to the Δ*thi3*-Δ*spe1*-Δ*pro2* strain and both strains do not show any notable decreases in the higher alcohols and volatile fatty acids, above those already observed for the single and double deletion strains, discussed above. It would seem that the deletion of *THI3* has the major impact and that additional deletions does not affect the Ehrlich pathway significantly.




A




B

**Fig. 5.5**. GC-FID analysis of the concentrations of the metabolites formed from the branched-chain amino acid isoleucine via the Ehrlich pathway. Panel A represents the higher alcohol, isoamyl alcohol, whilst panel B shows the results for the volatile branched-chain acid, iso-valeric acid. Results are the end-level amounts formed after cells were grown for one week in SCD5+++ medium. The graph shows the

differences between all the deletion strains and the consensus wild type. The experiments were performed in triplicate and the error bars are shown. Groupings denoting significant differences after conducting one-way ANOVA, followed by the Holm-Sidak post-test to determine statistical variability between the different strains, are indicated by the lettering above the columns. Groups B+ and C- are statistically not significantly different, but B+ differs statistically from C and C- differs statistically from B.

In an attempt to perturb all four reaction of the Ehrlich pathway, deletion strains Δ*bat2*-Δ*thi3*-Δ*hom2* and Δ*bat2*-Δ*thi3*-Δ*aad6* were created. These strains combined deletions of a transaminase (*BAT2*), a decarboxylase (*THI3*), and a dehydrogenase (*AAD6* and *HOM2*). It is also clear from the radar plot that these triple deletions seems to have had the biggest effect on the end-products of the Ehrlich pathway, especially the Δ*bat2*-Δ*thi3*-Δ*aad6* strain. The higher alcohols isobutanol and isoamyl alcohol decreased notably. Almost the same effect is seen with the Δ*bat2*-Δ*thi3*-Δ*hom2* strain. This triple deletion, however, has a smaller effect on the end levels of the volatile fatty acids, with a minor decrease for iso-butyric acid and no difference observed for iso-valeric acid when compared with wild type values. In contrast, the Δ*bat2*-Δ*thi3*-Δ*aad6* strain showed notable decreases for both of these compounds.

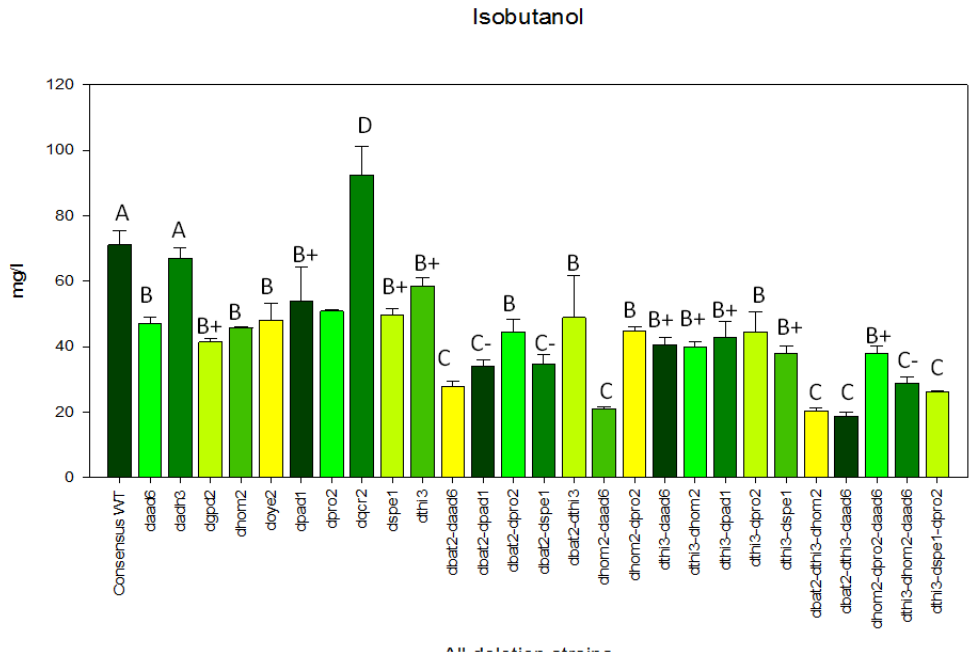
Interestingly there seems to be no change in the levels of the downstream metabolite octanoic acid for this strain, whilst levels of the closely related compound hexanoic acid showed some negative impact. This is not the case for the Δ*bat2*-Δ*thi3*-Δ*hom2* strain where both of these downstream compounds are notable affected by the triple deletion. It is also notable that both of these triple deletion strains effect the end levels of valeric acid to a large degree.

### 5.4.3 INVESTIGATING THE EFFECT OF DOUBLE AND TRIPLE DELETION STRAINS ON EHRLICH PATHWAY END METABOLITES

The above section highlighted the effect of combinatorial deletions on the overall aroma profile produced by the yeast during alcoholic fermentation. However, these strains were created to elucidate the roles of the selected genes in the Ehrlich pathway and it is therefore necessary to

focus on the end products of this pathway. In **Figures 5.5** end-level concentrations of the metabolites originating from isoleucine – isoamyl alcohol and iso-valeric are shown in mg/l, while **Figure 5.6** shows the final concentrations of isobutanol and iso-butyric acid – originating from valine. It is immediately clear that there are significant differences between the double and triple deletion strains and the single deletion- and wild type strains. However, to determine if a gene plays a direct or indirect role in the Ehrlich pathway, it is important to look at the fold-effect of the combinatorial deletion with regards to the corresponding single deletion strains. Various scenarios can arise: the additional deletion of a gene can cause an additive effect, surpassing that of both single deletion "parental" strains; the effect of additional deletions can also be epistatic or neutral and cause the double or triple deletion strains to have a fold effect similar to one or either "parental" strains; or the additional deletion can lead to a reversal of the effect the deletion of a single gene has on the concentrations of Ehrlich pathway end metabolites. Depending on the effect of the double or triple deletion strains, one can make informed deduction as to the role that a certain gene plays in the Ehrlich pathway, whether it exerts its effect via direct or indirect involvement in the pathway and possible substrate specificities.

In order to facilitate these observations the log2 fold decreases of the single, double and triple deletion strains with regards to the wild type for the end metabolites of the Ehrlich pathway were calculated and plotted against each other, but grouped according to "parental" and combinatorial strains. In **Figure 5.7** the deletions involving *BAT2* is shown. It is once again clear that the double deletions involving a transaminase and a decarboxylase, i.e. Δ*bat2*-Δ*pad1* (panel B), and Δ*bat2*-Δ*spe1* (panel D) show similar fold decreases with neutral effects seen for isoamyl alcohol and isobutanol, but slight additive effects for iso-valeric acid and notable additive effects for iso-butyric acid. This indicates that although the flux through the Ehrlich pathway towards the formation of higher alcohols seem to be controlled by Bat2p, the flux towards the formation of the volatile branched-chain acids seems to be less stringently controlled and downstream enzymes can exert an effect. This might also explain the relative lack of any effect seen with the transaminase-reductase deletion strain Δ*bat2*-Δ*pro2* (panel C).

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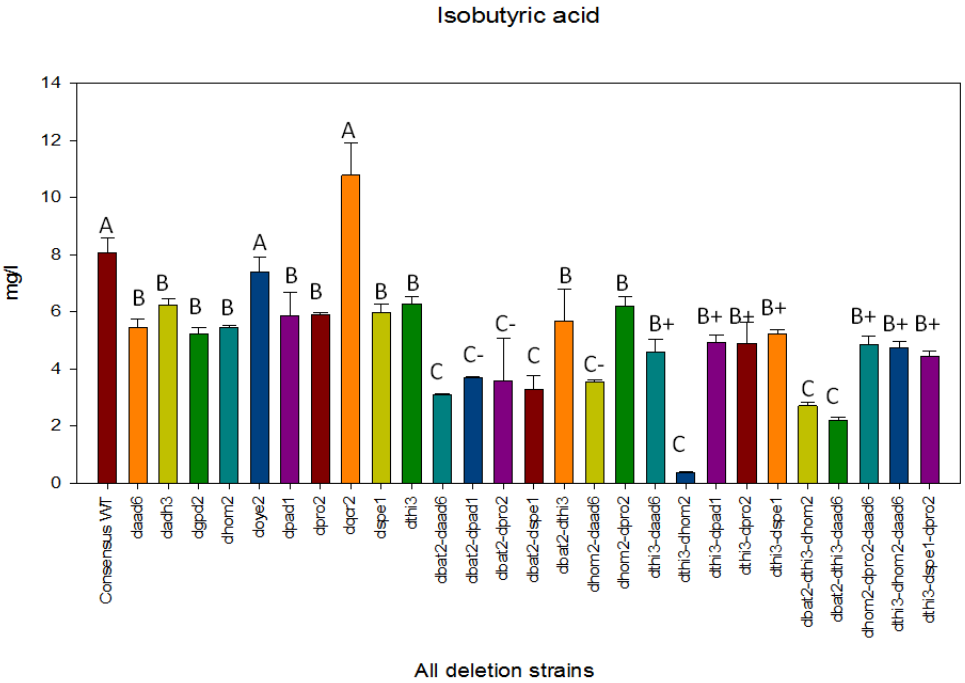


B

**Fig. 5.6**. GC-FID analysis of the concentrations of the metabolites formed from the branched-chain amino acid valine via the Ehrlich pathway. Panel A represents the higher alcohol, isobutanol, whilst panel B shows the results for the volatile branched-chain acid, iso-butyric acid. Results are the end-level amounts formed after cells were grown for one week in SCD5+++ medium. The graph shows the differences between all the deletion strains and the consensus wild type. The experiments were performed in

triplicate and the error bars are shown. Groupings denoting significant differences after conducting one-way ANOVA, followed by the Holm-Sidak post-test to determine statistical variability between the different strains, are indicated by the lettering above the columns. Groups B+ and C- are statistically not significantly different, but B+ differs statistically from C and C- differs statistically from B.

However, the other decarboxylase gene in combination with *BAT2*, Δ*bat2*-Δ*thi3* (panel E) has a unique profile and shows decreased negative effects towards all Ehrlich end metabolites, except iso-butyric acid, when compared with only the single *BAT2* deletion strain. This is harder to explain, but if *THI3* has a direct role in the Ehrlich pathway, then a positive feed-back loop can account for this phenomenon, where low levels of the end metabolites of the Ehrlich pathway are sensed, causing a increase in the flux through the pathway, by way of other, as yet unidentified enzymes. Mention was already made above of the seeming selectivity of the Δ*bat2*-Δ*aad6* strain towards valine and this can once again be observed in panel A, where only for the end products of this amino acid, the double deletion strain shows significant additive effects, whilst negative and neutral effects are seen on the levels of isoamyl alcohol and iso-valeric acid, respectively – the metabolites of isoleucine. This would indicate that *AAD6* plays a direct role in the Ehrlich pathway and shows an enhanced affinity for the compounds associated with valine metabolism.

The effect on the fold decrease in the levels of Ehrlich pathway end metabolites by double deletions in combination with *HOM2* are shown in **Figure 5.8**. Once again the selectivity of the *AAD6* gene for metabolites originating from valine is shown in panel A with the Δ*hom2*-Δ*aad6* strain, where large additive effects are observed for isobutanol and iso-butyric acid. This is once again a clear indication that *AAD6* plays a direct role in the Ehrlich pathway, especially with regards to valine metabolism. However, the combination of two dehydrogenases deleted has little effect on the fold decrease seen for isoamyl alcohol, although the decrease in the levels of iso-valeric acid is completely abrogated in this strain. The same effect for iso-valeric acid is seen in the dehydrogenase-reductase double deletion strain Δ*hom2*-Δ*pro2*, coupled to

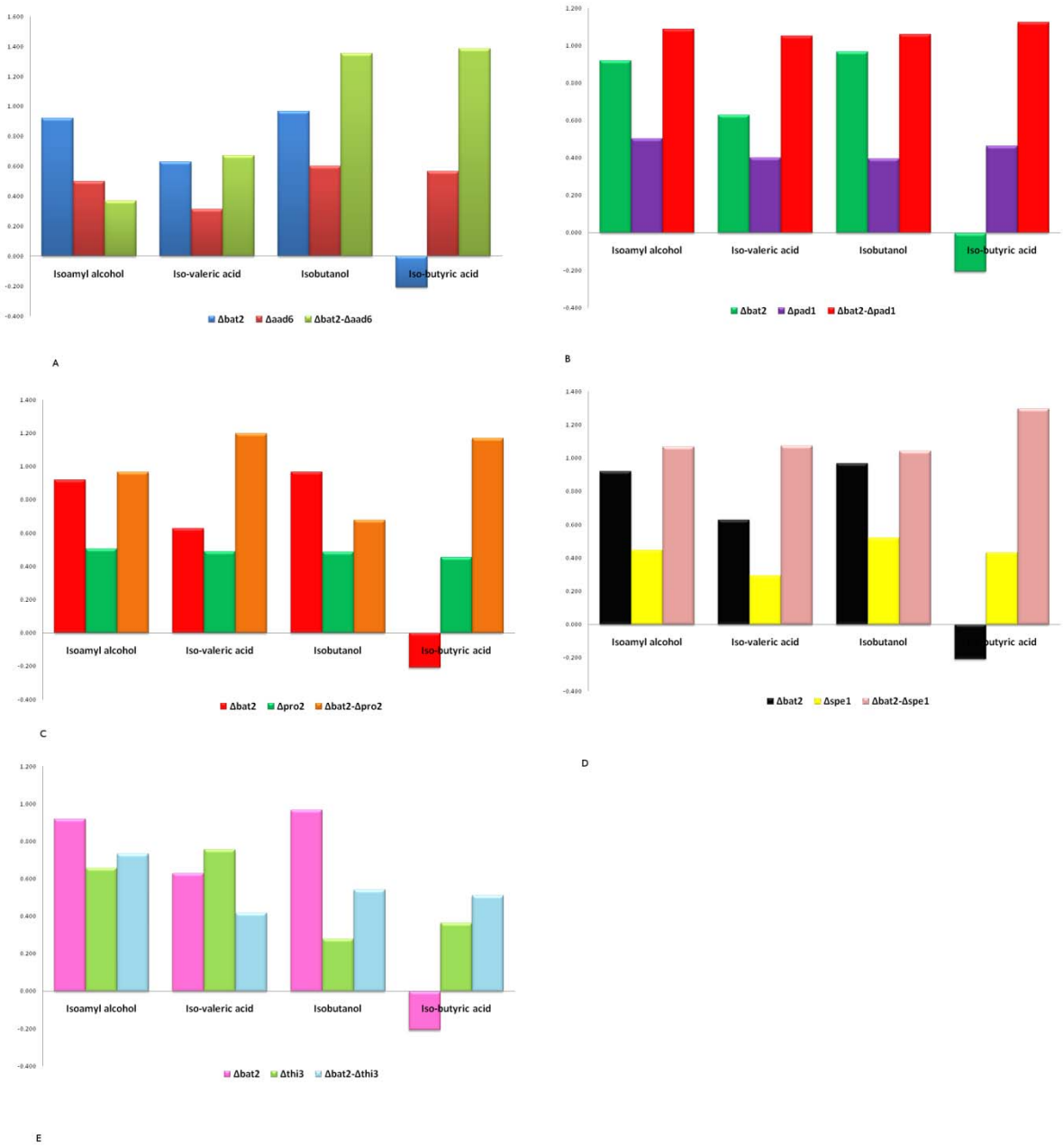


**Fig. 5.7**. The effect of double deletions with *BAT2* and various selected genes on fold decreases in the levels of the end metabolites of the Ehrlich pathway, isoamyl alcohol, iso-valeric acid, isobutanol and iso-butyric acid with regards to the consensus wild type levels are indicated by the bars as log2 values.

diminished fold decreases for isoamyl alcohol and iso-butyric acid and a neutral result for isobutanol. These results clearly indicate that *HOM2* plays an important role in the Ehrlich pathway and combinatorial deletions with a gene of lesser importance such as *PRO2*, causes the cell to compensate for the deletions by increasing the flux through the pathway using other enzymes capable of catalysing the reactions.

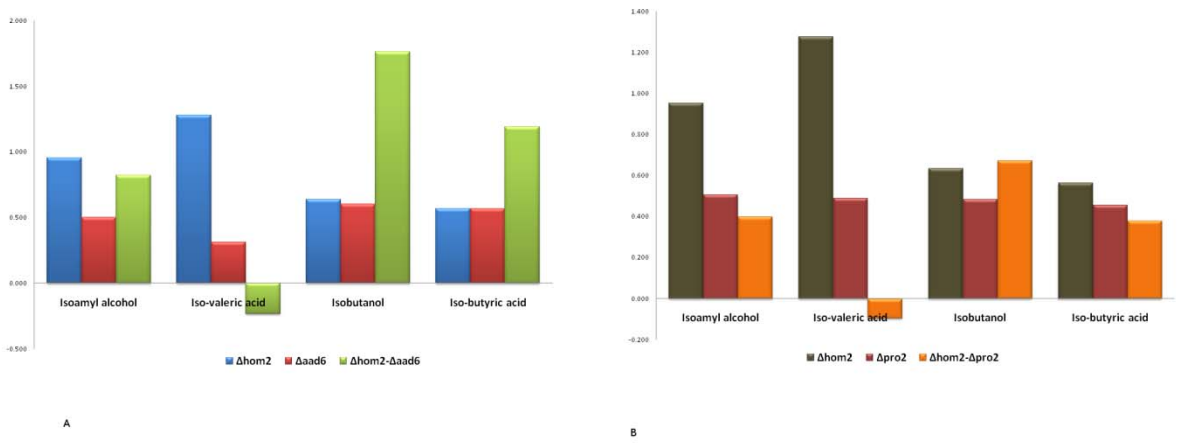


**Fig. 5.8**. The effect of double deletions with *HOM2* and various selected genes on fold decreases in the levels of the end metabolites of the Ehrlich pathway, isoamyl alcohol, iso-valeric acid, isobutanol and iso-butyric acid with regards to the consensus wild type levels are indicated by the bars as log2 values.

Deletion strains with a double decarboxylase deletions such as Δ*thi3*-Δ*pad1* and Δ*thi3*-Δ*spe1* have remarkably similar effects on the fold decreases of Ehrlich reaction end metabolites when compared to single deletion "parental" strains, as observed in **Figure 5.9**, panels C and E. As with the previously discussed Δ*bat2*-Δ*thi3* strain these double deletion strains show a clear additive effect on the fold decreases of isobutanol and iso-butyric acid, with dramatic negation of these fold decreases for the metabolites associated with isoleucine, i.e. isoamyl alcohol and iso-valeric acid. Whether this also indicates a preference of the thi3p towards valine related metabolites as was shown with aad6p remains a possibility, but the negative effects towards isoleucine related metabolites could once again indicate a positive feed-back loop used to regulate the flux through the pathway towards these end products. Similar effects seen with deletions of *THI3* with dehydrogenase (Δ*thi3*-Δ*aad6*) and reductase (Δ*thi3*-Δ*pro2*) genes, panels A and D, re-enforce these conclusions. Although *HOM2* is also thought to play a direct role in the Ehrlich pathway, deletion of this gene together with *THI3* has little impact on the fold decreases of the single deletion strains, with the exception of large additive effect seen with iso-butyric acid, once again indicating that all end metabolites of the Ehrlich pathway are not regulated similarly and various other unknown factors could still play a role the production of these compounds, not contained in the scope of this study.

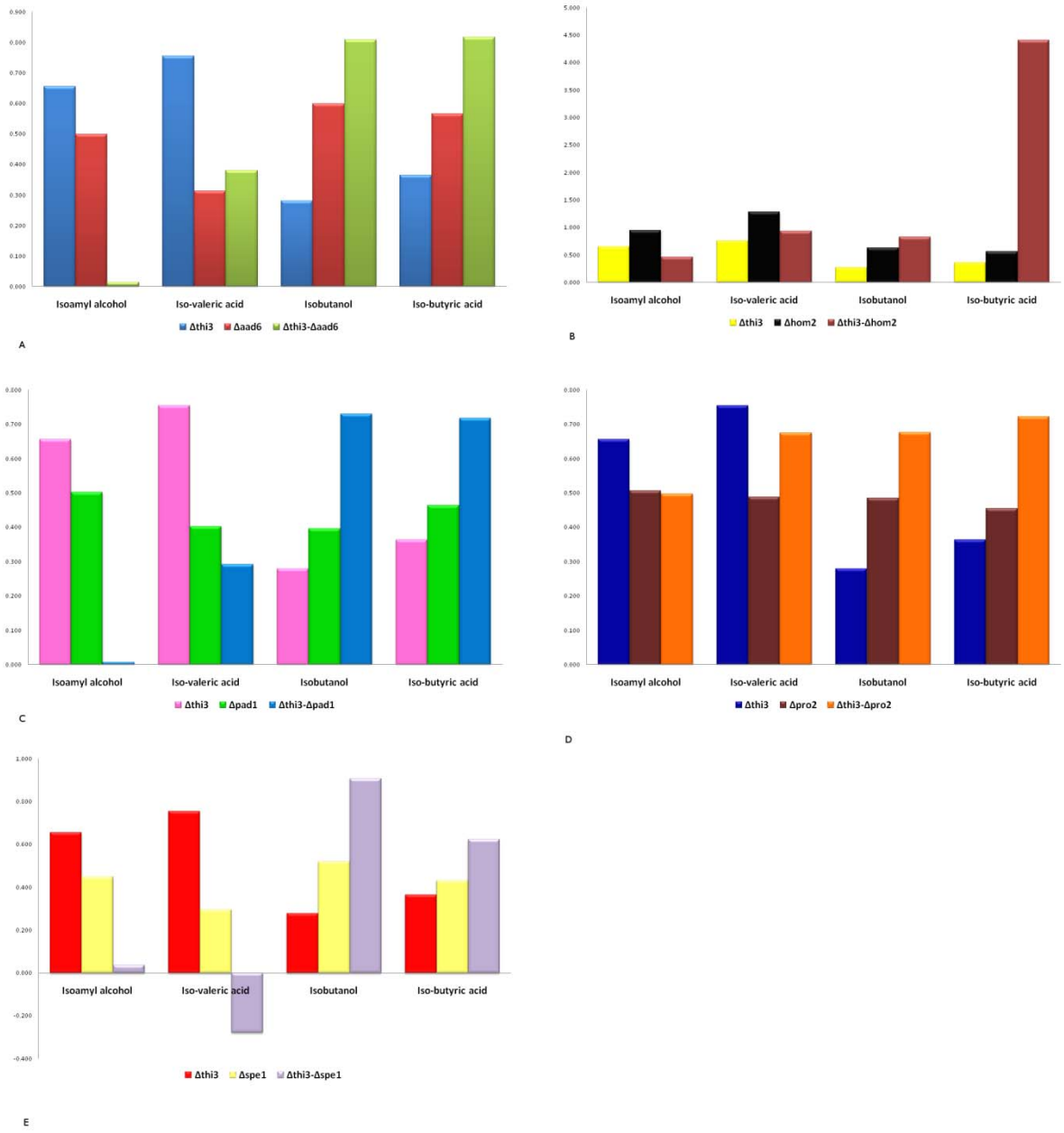


**Fig. 5.9**. The effect of double deletions with *THI3* and various selected genes on fold decreases in the levels of the end metabolites of the Ehrlich pathway, isoamyl alcohol, iso-valeric acid, isobutanol and iso-butyric acid with regards to the consensus wild type levels are indicated by the bars as log2 values.

In **Figure 5.10** the effect on the fold decreases in the levels of Ehrlich pathway end metabolites for the triple deletion strains are compared to their corresponding single deletion "parental" strains, as well as any double deletion strains. The Δ*hom2*-Δ*pro2*-Δ*aad6* strain (panel C) should be severely comprised in its ability to form higher alcohols and volatile fatty acids as all three

genes play a role in oxidation/reduction reactions. This is also reflected in **Figure 5.10**, where the triple deletion strain has an additive effect on the fold decreases of all Ehrlich reaction end metabolites when compared to single and double deletion strains, with the exception of the levels of isobutanol and iso-valeric acid produced by Δ*hom2*-Δ*aad6*. This is a clear indication of the importance of these three genes on the flux through the Ehrlich pathway and once again argues for a direct role of each of their gene products in this reaction.

The Δ*thi3*-Δ*spe1*-Δ*pro2* strain (**Figure 5.10**, panel E) should be severely comprised in its ability to form higher alcohols and volatile fatty acids as *THI3* and *SPE1* play a role in the decarboxylase reaction, whilst *PRO2* is involved in oxidation/reduction reactions within the Ehrlich pathway. As with the above mentioned strain Δ*hom2*-Δ*pro2*-Δ*aad6*, the deletion of these three genes has an additive effect on the fold decreases for the Ehrlich reaction end metabolites, with the exception of iso-valeric acid, which is unchanged from that of the single and double deletion "parental" strains. This is an indication of the important and direct role that each of these genes has on the flux through the Ehrlich pathway, even if the situation for iso-valeric acid could be interpreted differently.

Another triple deletion strain with a combination of deleted genes which are involved in both decarboxylase and oxidation/reduction reactions is Δ*thi3*-Δ*hom2*-Δ*aad6* (**Figure 5.10**, panel D). This strain contains one decarboxylase deletion in the form of Δ*thi3* and two dehydrogenase deletions represented by Δ*hom2* and Δ*aad6*. The results, however, are very different for this strain compared to the above mentioned Δ*thi3*-Δ*spe1*-Δ*pro2* strain. Here only a slight additive effect is seen in the fold decrease for the higher alcohol isobutanol, while those for the rest of the Ehrlich pathway end metabolites remain at levels comparable with single and double deletion "parental" strains. Once again the positive feed-back loop might play a role in re-establishing the flux through the Ehrlich pathway as the triple deletion causes a severe reduction in this regard. However, this is speculative and needs to investigated further.

In an attempt to perturb all four reaction of the Ehrlich pathway, deletion strains Δ*bat2*-Δ*thi3*-Δ*hom2* and Δ*bat2*-Δ*thi3*-Δ*aad6* were created. These strains combined deletions of a transaminase (*BAT2*), a decarboxylase (*THI3*), and a dehydrogenase (*AAD6* and *HOM2*). These strains are compared to their corresponding single and double deletion "parental" strains with regards to the fold decrease in levels of end metabolites of the Ehrlich pathway relative to the wild type strain. In **Figure 5.10**, panel A the results for the Δ*bat2*-Δ*thi3*-Δ*hom2* strain are shown, whilst panel B, represents data for the Δ*bat2*-Δ*thi3*-Δ*aad6* strain. It is clear from panel A that deletion of the three genes *BAT2*, *THI3* and *HOM2*, has significant additive effects on the fold decreases for all the end metabolites of the Ehrlich pathway, with the exclusion of iso-valeric acid (a compound that seems to be regulated somewhat independently of the rest of the pathway). Only in the case of iso-butyric acid could this triple deletion strain not match the huge negative impact seen with the deletion of *HOM2*, either alone or in combination with *THI3*. The picture is even more clear-cut with regards to the Δ*bat2*-Δ*thi3*-Δ*aad6* strain, where a major additive impact on the fold decreases for these metabolites are observed. Once again, as observed above, the deletion of the AAD6 gene has a far greater impact on the metabolites originating from valine.

## 5.5 CONCLUSIONS

In a previous study (Styger, *et al.*, this dissertation) a large scale screening experiment was conducted to search for genes whose deletion negatively impacted on the end-products of the Ehrlich pathway, i.e. the higher alcohols isoamyl alcohol and isobutanol, and the volatile fatty acids iso-valeric acid and iso-butyric acid. During the screening process, ten genes of interest were identified. This study lists the results of further in-depth analysis of certain of these strains.

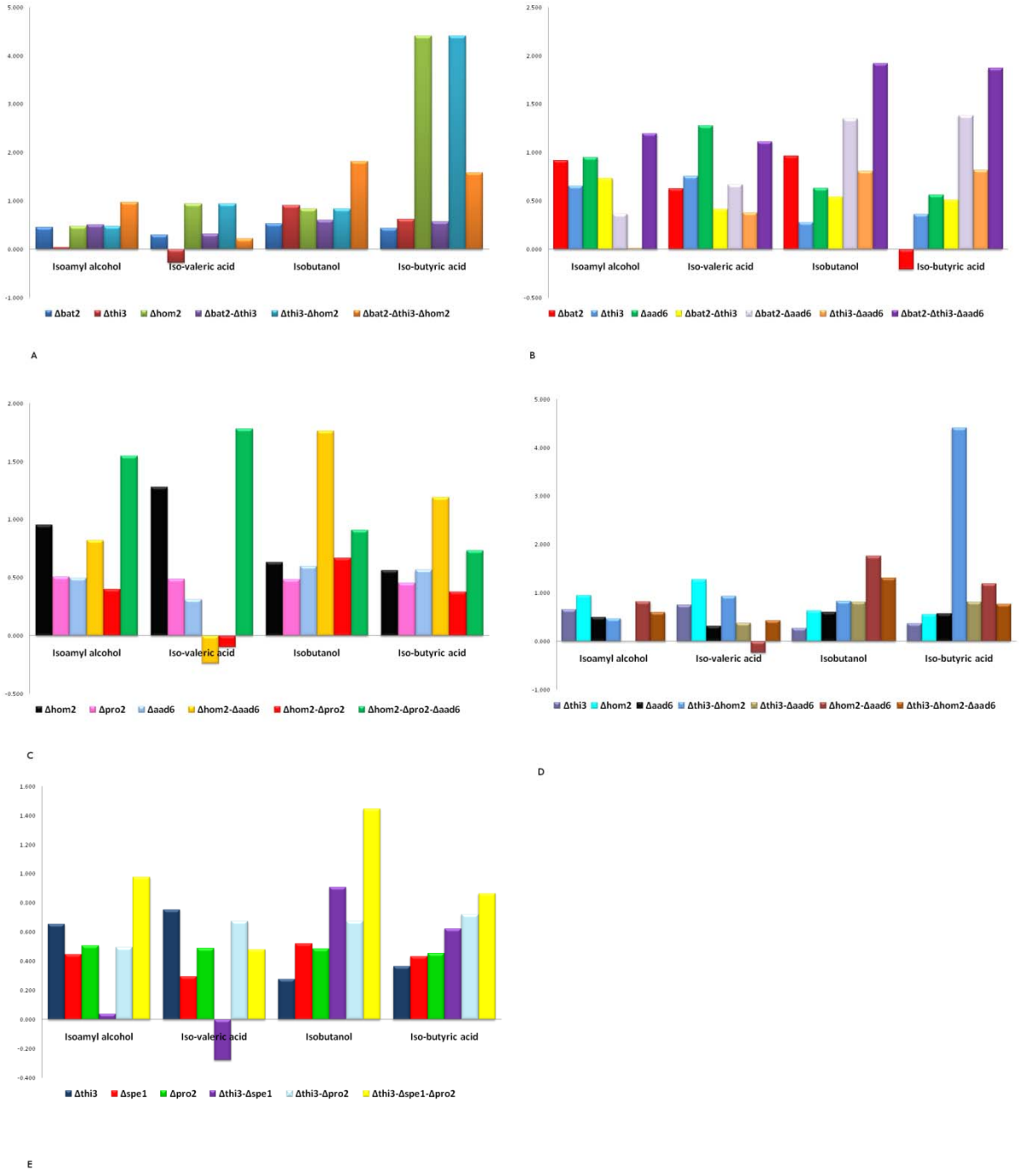


**Fig. 5.10**. The effect of various combinations of triple deletions with various selected genes on fold decreases in the levels of the end metabolites of the Ehrlich pathway, isoamyl alcohol, iso-valeric acid, isobutanol and iso-butyric acid with regards to the consensus wild type levels are indicated by the bars as log2 values.

The first area of concern was the ability of to replicate the findings of the initial screen by independently re-creating the same strains as those identified from the EUROSCARF library. In order to achieve this, alternative deletion cassettes were constructed (strains in the EUROSCARF library were constructed using Kanamycin resistance cassettes) and transformed into the BY4742 strain. It is clear from the results above that the independently constructed deletion strains showed the same properties as their corresponding EUROCARF deletion strains when comparing the effect of the particular deletion on the end levels of the compounds mentioned above. The only deletion strain that showed no difference to the wild type strain, i.e. in which the screening results could not be replicated, was the *QCR2* deletion strain. This gene encodes subunit 2 of the ubiquinol cytochrome-c reductase complex, which is a component of the mitochondrial inner membrane electron transport chain. It is thus difficult to envisage a role for this gene in the production of higher alcohols and volatile fatty acids via the Ehrlich pathway. It was initially thought that deletion of this gene perturbed the redox homeostasis of the cell, leading to compensation through changes in the flux through the Ehrlich pathway. However, it would seem as if this strain was incorrectly identified during the initial screening experiment and again underlines the importance of independently confirming results from such large scale screening experiments with strain libraries.

Overall the over-expression of neither of the remaining nine genes resulted in any major effects on the Ehrlich pathway. Rather than interpreting this result to mean that none of these genes are essential in the Ehrlich pathway, one can hypothesise that the cell will place a cap on the maximum amounts of these compounds that can be produced – it is important to keep in mind that the cell exports these compounds into the growth medium and therefore it would be energy inefficient to make very large quantities of compounds that will probably be lost to the cell. Information on the regulation of this and other aroma production pathways is still incomplete, but it is not inconceivable that the production of higher alcohols and volatile fatty acid may be tightly regulated. However, the most likely explanation is that the particular genes in the over-expression strains are only under control of the constitutive PGK promoter and that any

perturbations thus caused are not in of the same magnitude as a complete deletion of the gene as in the deletion strains. Therefore it was not attempted to construct any double or triple over-expression strains.

The six most promising genes, i.e. *AAD6*, *HOM2*, *PAD1*, *PRO2*, *SPE1* and *THI3* were further investigated by constructing double and triple deletion combinations of these genes and the transaminase gene *BAT2*. Whilst all the double deletion strains showed a decrease in the production of isobutanol, isoamyl alcohol, iso-butyric and iso-valeric acid these effects were not always substantially greater than the effects seen with single deletions of these genes. It would seem, however, that only double deletion strains in combination with Δ*bat2* and the Δ*hom2*-Δ*aad6* strain effected the production Ehrlich pathway end metabolites notably more than the corresponding single deletion strains.

These results seem to clearly indicate that the *BAT2* gene is the dominant gene in these deletion strains. This would mean that the first reaction of the Ehrlich pathway – the transaminase step – in principle becomes the rate-limiting step of this pathway, as various other secondary deletions of reaction further downstream seem not to have such a large impact on the production of higher alcohols and volatile fatty acids. It could also indicate that whilst the transaminase reaction is highly specific for *BAT2* and to a much lesser extent *BAT1* [51], the other reactions of the Ehrlich pathway are not as specific. To see if this hypothesis was true, triple deletion strains were constructed where the effect of multiple combinations of deletions on the Ehrlich pathway, could be investigated.

Interestingly enough, the triple deletion strains in combination with BAT2, i.e. Δ*bat2*-Δ*thi3*-Δ*aad6* and Δ*bat2*-Δ*thi3*-Δ*hom2* have the biggest impact on the end metabolites of the Erlich pathway, with the exception of isoamyl alcohol and iso-valeric acid. The strain deleted for two

dehydrogenases and a reductase - Δ*hom2*-Δ*pro2*-Δ*aad6* had a greater effect on the levels of these two compounds. As both these compounds are catabolised from the branched-chain amino acid leucine, it could be that there is some sort of preferential usage of these amino acids and the deletion of the genes encoding for these enzymes thus has a greater impact on the metabolism of this amino acid. In other cases a bigger impact was seen for metabolites originating from valine, suggesting that it is possible that these enzymes, though acting in a promiscuous manner, still maintain some form of substrate specificity.

It is clear from the above results that BAT2p has been once again confirmed to play a role as a branched-chain transaminase in the Ehrlich pathway. The sensitivity of the Ehrlich pathway to cellular redox homeostasis has been shown with the effects of deletions of *GPD2* and *ADH3* on the pathway. There is also good evidence that identifies THI3p as the decarboxylase that catalyses the conversion of the α-keto acid into an aldehyde and that AAD6p and HOM2p play a direct biochemical role in the Erlich pathway by reducing or oxidising the aldehyde to produce either a higher alcohol or a volatile fatty acid. The other candidate genes investigated, i.e. *PAD1*, *PRO2* and *SPE1* all seem to play a direct role in the Ehrlich pathway, although to a lesser extent than the above genes. It is possible that these genes play the role of promiscuous genes – capable of catalysing a wide range of substrates. Furthermore the data indicates that certain levels of substrate selectivity exists within the Ehrlich pathway with the Aad6p showing a preference for metabolising the compounds originating from valine. However, further biochemical studies are necessary to show that this is indeed taking place in yeast strains during wine making conditions. This study has contributed to the further elucidation of the Ehrlich pathway, but also indicates that this aspect of yeast metabolism is still poorly understood and further investigation is needed to elucidate the physiological role of higher alcohol metabolism, the genes and enzymes involved in the pathway and the regulation thereof.

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# Chapter 6

## General discussion and conclusions

# 6. GENERAL DISCUSSION AND CONCLUSIONS

## 6.1 CONCLUDING REMARKS

Wine science, as a mostly applied science describing a complex environment, has been focusing in the past on establishing correlations between specific practices and specific outcomes. Many studies have been trying to establish the effect of individual parameters / practices on final wine quality. However, because of the complexity of the topic, these studies have mostly failed in establishing true causality. With the advancement of science in the past decade or so, the science of wine and winemaking has also taken major leaps forward. Gone are the days of “the effect of” type of study, and more and more system-based tools are being used, with high throughput experiments becoming the norm [1].

This is in particular the case for studies relating to the impact of yeast, since this organism has been and continues to be a preferred scientific model system. According to Borneman, *et al.*, 2007 [2] yeast research is poised at an important junction between applied and fundamental research, and the wine industry can reap enormous benefits from the next level of biological research, i.e. systems biology [2]. Already studies combining transcriptomic data with classical wine chemistry and advanced chemometric data processing are opening interesting new avenues of research [3].

The original scope of this study was to compare different transciptome analysis methods and to determine whether one could compare data from two such disparate approaches. The first technique used was oligo-cDNA microarrays. This technique is still widely used, although the Affymetrix chip has taken precedence over oligo-cDNA microarrays. The second transcriptome analysis technique investigated was Serial Analysis of Gene Expression (SAGE). This is a technique developed in 1995 by Velculescu *et al.* [4, 5]. Unfortunately the

labour-intensive SAGE technique could not be positively implemented in our laboratory conditions and the project was abandoned and it was decided to shift the focus of this study.

The cDNA microarray dataset nevertheless remains a valid representation of the changes the yeast transcriptome undergoes during the course of a model wine fermentation. The data concurs with other published studies on the transcriptome analysis of wine yeast strains [6-9] and stands as an important dataset with which to compare data from newer studies using the Affymetrix system [3]. The dataset was also valuable in identifying genes that were induced during stationary phase, the promoters of which were used as stationary phase inducible promoters to drive the transcription of various genes involved in flocculation [10].

Although the pathways by which most aroma compounds are produced are well known, little is known about the genes involved and the regulation of these genes [3]. Previous work also highlighted the seeming interconnectedness of the pathway – changes in the production of a certain set of aroma compounds invariably had knock-on effects on other, seemingly unrelated aroma compounds [11]. Thus the main aim of the study was to use an “old fashioned” screening approach to systematically identify genes whose products could be involved in aroma compound production during a model wine fermentation. This unbiased approach would give us the chance to identify genes that might not have been considered when using a different, more directed approach. One caveat, however, of this unbiased approach is the difficulty of defining the best criteria for a phenotype for which to screen.

Previous work on the catabolism of the branched-chain amino acids into aroma important compounds such as higher alcohols and volatile branched-chain fatty acids revealed that the first step in the catabolism reaction, the so-called Ehrlich reaction [12], involved the

deamination of the branched-chain amino acid into an α-keto acid. This reaction was catalysed by the mitochondrial and cytosolic branched-chain amino acid aminotransferases (BCAATases) encoded by the *BAT1* and *BAT2* genes [11, 13-17]. Deletion of these genes resulted in significantly lower levels of the higher alcohols and related compounds [11]. Thus our phenotype for the large scale screen would involve the production of these compounds and thus we chose the participants of the screen to fit in with the biochemical reactions within the Ehrlich pathway, i.e. decarboxylases, reductases and dehydrogenases. It has been suggested that an alcohol dehydrogenase may catalyze this reductive reaction and an aldehyde dehydrogenase the oxidation reaction [18, 19]. Some studies postulate that the pyruvate dexarboxylase genes - *PDC1*, *PDC5* and *PDC6*, may play a part in this reaction, but they are not essential [20, 21]. Other possible decarboxylase genes that could be involved are *KID1/THI3* and *ARO10* [18, 19, 22].

Ultimately ten genes were chosen from the hundred or so that were initially screened. The criteria of selection were as follows: deletion of possible candidate genes had to lead to large decreases in the production of higher alcohols such as isoamyl alcohol and isobutanol, associated with decreases in either iso-valeric acid or iso-butyric acid. The genes that best matched the criteria were further evaluated. Interestingly none of the pyruvate decarboxylase genes were identified with the initial screen and the only alcohol dehydrogenase that made the cut was *ADH3*. The reason for this could perhaps be that these gene families can complement one another and thus one would not pick up individual members. Only those genes which individually have a major impact on the system will be selected. Of the multitude of aldehyde dehydrogenases, only the aryl alcohol dehydrogenase *AAD6* was identified. However, *THI3* was amongst the genes identified by the initial screen, and the *ARO10* deletion strain would not grow under our conditions. The difference in growth conditions, with small flasks of minimal media for the screening compared to chemostat cultures for some of the other studies might explain why certain genes were not picked up in

our screening process. It should be remembered that wine fermentation is a batch process, and that the aim was to focus on genes that would significantly impact in such conditions. It is also interesting to note that differences in the growth conditions of the yeast cell could favour certain gene products over others to play a part in aroma metabolism.

Of the ten genes finally retained, several do not play a direct role in the Ehrlich pathway, especially *GPD2* and *ADH3*. Others that seem to be indirectly involved are *OYE2* and *QCR2.* It has been speculated that higher alcohols and volatile fatty acids are formed as part of the yeast cell's redox maintenance, as the formation of a higher alcohol involves reduction and that of a fatty acid, oxidation [23, 24]. Some authors, however, believe that the cell produces enough other redox equivalents such as glycerol, acetate, acetaldehyde and succinate to fulfil this role [25]. However, from the data presented in this study it is clear that the Ehrlich pathway is indeed sensitive to the redox maintenance of the cell. One would thus assume that deletion of a gene that is very important in the global cellular maintenance of redox homeostasis such as *GPD2* or *ADH3*, would reflect differentially on a redox sensitive pathway, i.e. affecting the production of metabolites whose metabolism involves redox co-factors such as $NAD^+$ or NADH, so that the production of compounds that can help restore the redox balance be boosted in favour of other metabolites whose production would further imbalance the system. The redox duality of the Erhlich pathway, where an aldehyde can be reduced to form a higher alcohol, with the concomitant formation of $NAD^+$, or be oxidised to form a branched-chain volatile fatty acid, as well as $NADH+H^+$ would seem the ideal target for such regulation. However, deletion of both *GPD2* and *ADH3* has the same effect on both higher alcohols and volatile fatty acids – a decrease in production. This seems counter-intuitive, but might be explained by the fact that both these end products are exported from the cell into the surrounding medium and as such may not represent an accurate picture of cellular redox homeostasis at that particular time point.

The six most promising genes, i.e. *AAD6*, *HOM2*, *PAD1*, *PRO2*, *SPE1* and *THI3* were further investigated by constructing double and triple deletion combinations of these genes and the transaminase gene *BAT2*. Whilst all the double deletion strains showed a decrease in the production of Ehrlich related compounds these effects were not always substantially greater than the effects seen with single deletions of these genes. These results seem to clearly indicate that the *BAT2* gene is the dominant gene in these deletion strains. This would mean that the first reaction of the Ehrlich pathway – the transaminase step – in principle becomes the rate-limiting step of this pathway, as various other secondary deletions of reaction further downstream seem not to have such a large impact on the production of higher alcohols and volatile fatty acids. Triple deletion strains were constructed where the effect of multiple combinations of deletions on the Ehrlich pathway could be investigated. It was found that the triple deletion strains in combination with BAT2, i.e. Δ*bat2*-Δ*thi3*-Δ*aad6* and Δ*bat2*-Δ*thi3*-Δ*hom2* had the biggest impact on the end metabolites of the Ehrlich pathway, with the exception of isoamyl alcohol and iso-valeric acid. The strain deleted for two dehydrogenases and a reductase - Δ*hom2*-Δ*pro2*-Δ*aad6* had a greater effect on the levels of these two compounds. From the above results it seems clear that of the six candidate genes that were further investigated, *AAD6*, *HOM2* and *THI3* play a direct role, together with *BAT2*, in the Ehrlich pathway, whilst the others – *PAD1*, *PRO2* and *SPE1* – are most probably promiscuous enzymes capable of catalyzing their specific reactions with a broad range of substrates. However, the fact remains that deletions of these genes has a significant negative impact on the production of higher alcohols and related compounds.

It has long been recognised that the old adage that enzymes are specific to their substrates and the reactions that they catalyze is not as clear-cut as first imagined. Enzymes can act promiscuously in various ways, i.e. condition promiscuity – where the enzyme shows

catalytic activity in environmental conditions different from their natural state; substrate promiscuity – where enzymes have a broad range of substrates that they can catalyze; and catalytic promiscuity – where the enzyme is able to catalyze distinctly different chemical reactions [26]. Whilst the first and latter scenario does not seem to be involved in this case – all of the candidate genes were initially included in the screen due to the nature of their catalytic reactions – the manner in which they can come about is the same. Most often promiscuity is due to point mutations in the protein. These can be propagated through a process known as gene sharing – a period in which a single enzyme must serve both its original function and a new function that has become advantageous to the organism, with subsequent gene duplication allowing one copy to maintain the original function, while the other diverges to optimize the new function [27, 28]. Alternatively, the mutations could be neutral, i.e. they could have no impact on the protein's ability to perform its primary biological function and only when conditions change so that selections favour new "promiscuous" functions will the protein evolve naturally [28, 29].

However, it seems unlikely that the promiscuous behaviour seen with these candidate genes are simply due to point mutations. These genes mostly have well-defined primary functions and it would seem superfluous for the cell to maintain an array of enzymes capable of catalyzing the various reactions that make up the Ehrlich pathway. Alternatively, this might also be an indication of the relatively recent emergence of this pathway, in evolutionary terms. It is interesting to speculate that with the selection of *S. cerevisiae* from a naturally occurring micro-organism to its present day role as a very specific narrow-range industrial workhorse, many cellular adaptations had to made. It is possible that the production of the higher alcohols, isoamyl alcohol and isobutanol, as well as the volatile fatty acids, iso-valeric- and iso-butyric acid via the Ehrlich pathway became necessary as the cell adapted to its new role and rather than "invent" a new range of enzymes to fulfil this role, it was easier to use the promiscuity of these candidate genes in order to achieve this. Indeed some

authors have hypothesised that this kind of flexible metabolome involves the well-defined linear pathways cross wired in a variety of unexpected ways. Evolution may then capitalise on these cross-wirings, as a way of adaptive plasticity (i.e., with no genetic changes to begin with), to generate new metabolic capabilities [30]. Another way of looking at enzyme promiscuity and the emergence of new metabolic properties is to divide the metabolism of an organism into three compartments i.e., basic integrated metabolism – involving pathways which had been evolutionary selected for a very long time and where further optimisation was not possible; supporting metabolism – where pathways are less integrated and give rise to a range of almost interchangeable metabolites that contribute vitally to the physical environment; and lastly speculative metabolism – where molecules with specific potent biomolecular activities are formed from pathways where selection forces may change rapidly and therefore ones that can be formed at an energetically low cost [31, 32]. Thus it is possible that the Ehrlich pathway could have emerged due to the promiscuous nature of various enzymes.

In summary this work shows that BAT2p has been once again confirmed to play a role as a branched-chain transaminase in the Ehrlich pathway. The sensitivity of the Ehrlich pathway to cellular redox homeostasis has been shown with the effects of deletions of *GPD2* and *ADH3* on the pathway. There is also good evidence that identifies THI3p as the decarboxylase that catalyses the conversion of the α-keto acid into an aldehyde and that AAD6p and HOM2p play a direct biochemical role in the Erlich pathway by reducing or oxidising the aldehyde to produce either a higher alcohol or a volatile fatty acid, whilst the others – *PAD1*, *PRO2* and *SPE1* – are most probably promiscuous enzymes capable of catalyzing their specific reactions with a broad range of substrates. However, further biochemical studies are necessary to show that this is indeed taking place in yeast strains during wine making conditions.

## 6.2 FUTURE WORK

As in all scientific studies, this study leaves at least as many questions as answers:

a. Biochemical and enzymatic analysis of the ten candidate genes to determine if they are in fact capable of catalysing any of the reactions of the Ehrlich pathway
b. Creation of a GC-MS detection method in order to detect intermediates of the Ehrlich pathway, like the keto-acids and aldehydes, and not just the end products such as higher alcohols and volatile fatty acids. This will provide a better indication of the flux through the pathway

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# Appendix

Lilly M., Bauer F.F., Styger G., Lambrechts M.G. & Pretorius I.S., (2006) The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. *FEMS Yeast Research*. **6**(5) 726-743

# The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates

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## Abstract

In *Saccharomyces cerevisiae*, branched-chain amino acid transaminases (BCAAT ases) are encoded by the *BAT1* and *BAT2* genes. BCAATases catalyse the transfer of amino groups between those amino acids and α-keto-acids. α-Keto-acids are precursors for the biosynthesis of higher alcohols, which significantly influence the aroma and flavour of yeast-derived fermentation products. The objective of this study was to investigate the influence of *BAT*-gene expression on general yeast physiology, on aroma and flavour compound formation and on the sensory characteristics of wines and distillates. For this purpose, the genes were over-expressed and deleted in a laboratory strain, BY4742, and overexpressed in an industrial wine yeast strain, VIN13. The data show that, with the exception of a slow growth phenotype observed for the *BAT1* deletion strain, the fermentation behaviour of the strains was unaffected by the modifications. The chemical and sensory analysis of fermentation products revealed a strong correction between *BAT* gene expression and the formation of many aroma compounds. The data suggest that the adjustment of *BAT* gene expression could play an important role in assisting winemakers in their endeavour to produce wines with specific flavour profiles.

## Introduction

Winemakers employ a variety of techniques and tools to produce wines with specific flavour profiles. One of these tools is the choice of yeast strain to conduct fermentation. The wine yeast *Saccharomyces cerevisiae* brings forth the major changes between grape must and wine, modifying aroma, flavour, mouth-feel, colour and chemical complexity. During fermentation, *S. cerevisiae* produces a range of minor but sensorially important volatile metabolites that gives wine its vinous character. These volatile metabolites, which are derived from the sugar and amino acid metabolism of wine yeast, include esters, carbonyls, volatile fatty acids, sulphur compounds and higher alcohols (for recent reviews, see Lambrechts & Pretorius, 2000; Swiegers & Pretorius, 2005; Swiegers *et al*., 2005).

The term 'higher alcohol' refers to alcohols that possess more than two carbon atoms and have a higher molecular weight and boiling point than ethanol. Higher alcohols, also known as fusel alcohols, are quantitatively the largest group of aroma compounds in many alcoholic beverages (Amerine *et al*., 1980). They are identified by a strong, pungent smell and taste and can have a significant effect on the sensorial quality and character of wine and brandy (Rapp & Mandery, 1986; Pretorius & Høj, 2005; Swiegers & Pretorius, 2005; Swiegers *et al*., 2005). Higher alcohols are composed of aliphatic and aromatic alcohols (Nykänen & Nykänen, 1977). The aliphatic alcohols include propanol, isobutanol, active amyl alcohol and isoamyl alcohol, while phenylethyl alcohol is considered to be one of the most important aromatic alcohols contributing to wine flavour. The sulphur-containing alcohols, for example methionol, might also have a strong influence on taste and flavour (Lambrechts & Pretorius, 2000). Isoamyl alcohol, active amyl alcohol and isobutanol are also known as branched-chain alcohols because they are the degradation products of the branched-chain amino acids, leucine, isoleucine and valine (Lambrechts & Pretorius, 2000).

During alcoholic fermentation, *S. cerevisiae* produces higher alcohols that can influence the flavour of the end-

product. The taste threshold levels of some of the higher alcohols in wine and model solutions indicate that differences in the amounts of higher alcohols could influence the taste of wines (Rankine, 1967).

The higher alcohols produced by yeast can originate from the degradation of imported branched-chain amino acids (BCAA) or from endogenous biosynthesis. The BCAA uptake in *S. cerevisiae* is mediated by at least three transport systems, i.e. the general amino acid permease Gap1p, the BCAA permease Bap2p, and one or more unknown permeases (Didion *et al.*, 1996). The amino acids are converted to their corresponding α-keto acids by transamination (leucine to α-ketoisocaproic acid, valine to α-ketoisovaleric acid, and isoleucine to α-keto-β-methylvaleric acid) (Dickinson & Norte, 1993; Dickinson *et al.*, 1997). This transamination reaction is catalysed by mitochondrial and cytosolic branched-chain amino acid transferases (BCAATases) encoded by the *BAT1* and *BAT2* genes, respectively (Eden *et al.*, 1996, 2001; Kispal *et al.*, 1996). Alternatively, these α-keto acids can be generated through the *de novo* synthesis pathway from glucose (Dickinson *et al.*, 1997). Branched-chain alcohols are then synthesized from the corresponding α-keto acids by decarboxylation and reduction (Dickinson *et al.*, 2000, 1997). The first reaction is catalysed by a decarboxylase, which converts the α-keto acid to the corresponding branched-chain aldehyde through the removal of one carbon atom (Dickinson *et al.*, 1997). This is followed by a reaction catalysed by an alcohol dehydrogenase, which leads to the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol (Derrick & Large, 1993). Alternatively, the aldehyde may be oxidized in a $NAD^+$-dependent reaction to an acid (Fig. 1). Therefore, it is likely that the redox state of a yeast cell is important for determining the fate of the α-keto acid.

The physiological function of higher-alcohol production by yeast is unclear, although many hypotheses have been postulated. It has been suggested that, physiologically, oxidative deamination provides the yeast with a mechanism for obtaining nitrogen when its pool has become depleted (Vollbrecht & Radler, 1973). A second hypothesis has proposed that higher-alcohol production contributes to the maintenance of the redox balance in the cell because the final reduction step in higher-alcohol production involves the reoxidation of $NADH+H^+$ to $NAD^+$ (van Dijken & Scheffers, 1986; Quain, 1988; Zoecklein *et al.*, 1995). However, it has also been stated that there appears to be enough acetaldehyde to maintain the redox balance and that the formation of higher alcohols is not considered to be an important means for the reoxidation of NADH (Boulton *et al.*, 1995). Finally, it has been suggested that higher-alcohol production might act as a detoxification process for the intracellular medium of α-keto acids and aldehydes, or as a means of regulating the metabolism of amino acids (Ribéreau-Gayon *et al.*, 2000).

The objective of this study was to gain a greater understanding of the roles that the *BAT1*- and *BAT2*-encoded BCAATases of *S. cerevisiae* play during alcoholic fermentation, and of their influence on the concentration of higher alcohols and the flavour profile of wine and distillates.

## Materials and methods

### Microbial strains, media, and culture conditions

All yeast and bacterial strains used in this study and their relevant genotypes are listed in Table 1. *Escherichia coli* cells were grown in Luria–Bertani broth (Biolab, Midrand, South Africa) at 37 °C (Sambrook *et al.*, 1989). *Saccharomyces cerevisiae* cells were grown at 30 °C in a synthetic medium SCDSM [containing 0.67% yeast nitrogen base without amino acids (Difco, Sparks, MD), 0.13% of an amino acid stock solution (Ausubel *et al.*, 1994) lacking valine and isoleucine and supplemented with 0.5% glucose and 400 μg $L^{-1}$ sulfometuron methyl (Dupont, Wilmington, DE) per mL dissolved in *N*-,*N*-dimethylformamide]. *Saccharomyces cerevisiae* cells were also grown in a rich medium, YPD (containing 1% yeast extract, 2% peptone and 2% glucose). The SCD and SCR media contained 0.67% yeast nitrogen base without amino acids plus 60 μg $mL^{-1}$ leucine, 20 μg $mL^{-1}$ uracil, 20 μg $mL^{-1}$ histidine and 30 μg $mL^{-1}$ lysine, as well as either 4% glucose (for SCD) or 4% raffinose (for SCR). $SCD^{complete}$ and $SCR^{complete}$ contained 0.13% amino acid stock and $SCD^{leu,ile,val}$ and $SCR^{leu,ile,val}$ media

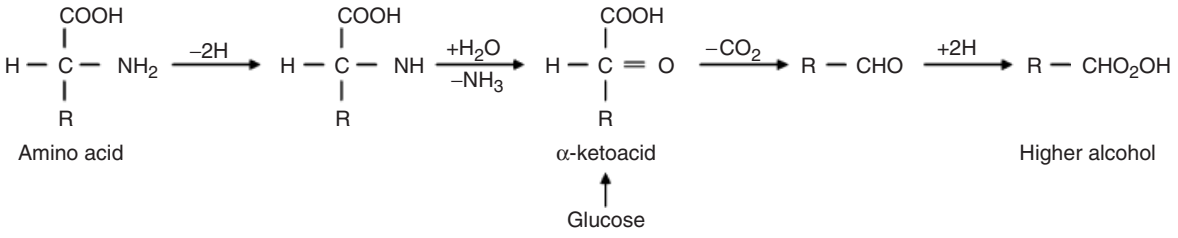


**Fig. 1.** The Ehrlich pathway for higher-alcohol production (Ehrlich, 1904).

**Table 1.** Microbial strains and plasmids used in this study

| Strain or plasmid | Genotype or construct | Reference or source |
|---|---|---|
| *Escherichia coli* DH5α | F′ *endA1 hsdR17* ($r_k^-$ $m_k^+$) *supE44 thi-1 recA1 gyrA* ($Nal^r$) *relA1*Δ(*laclZYA-argF*)*U169 deoR* [F80d*lac* DE(*lacZ*)M15] | GIBCO-BRL/Life Technologies |
| *Saccharomyces cerevisiae* | | |
| Industrial strain | | |
| VIN13 | Commercial wine yeast strain | Anchor Yeast, Cape Town, South Africa |
| Transformants | | |
| VIN13(pBAT1-s) | *SMR1-140 PGK1$_P$-BAT1-PGK1$_T$* | This study |
| VIN13(pBAT2-s) | *SMR1-140 PGK1$_P$-BAT2-PGK1$_T$* | This study |
| Laboratory strain | | |
| BY4742 | *MATα his3*Δ*leu2*Δ*lys2*Δ*ura3*Δ | Euroscarf |
| Transformants | | |
| BY4742(pBAT1-s) | *MATα his3*Δ*leu2*Δ*lys2*Δ*ura3*Δ*SMR1-140 PGK1$_P$-BAT1-PGK1$_T$* | This study |
| BY4742(pBAT2-s) | *MATα his3*Δ*leu2*Δ*lys2*Δ*ura3*Δ*SMR1-140 PGK1$_P$-BAT2-PGK1$_T$* | This study |
| BY4742Δbat2 | *MATα his3*Δ*leu2*Δ*lys2*Δ*ura3*Δ*bat2*Δ | Euroscarf |
| BY4742Δbat1 | *MATα his3*Δ*leu2*Δ*lys2*Δ*ura3*Δ*bat1*Δ | This study |
| Plasmids | | |
| pHVXII | *bla LEU2 PGK1$_P$-PGK1$_T$* | Volschenk *et al.* (1997) |
| pDLG42 | *bla SMR1-140* | This study |
| pBAT1-s | *bla SMR1-140 PGK1$_P$-BAT1-PGK1$_T$* | This study |
| pBAT2-s | *bla SMR1-140 PGK1$_P$-BAT2-PGK1$_T$* | This study |

contained the same as $SCD^{complete}$ and $SCR^{complete}$, respectively, with an additional 100 mg $L^{-1}$ leucine, isoleucine and valine. To $SCD^{leu}$ lysine, histidine and uracil were added plus 150.38 mg $L^{-1}$ leucine. $SCD^{ile}$ also contained lysine, histidine, uracil and leucine plus 134.98 mg $L^{-1}$ isoleucine. To the $SCD^{val}$ medium lysine, histidine, uracil and leucine were added as well as 225.94 mg $L^{-1}$ valine. Solid media contained 2% agar (Difco).

*Saccharomyces cerevisiae* strains were precultured in 50 mL SCD or SCR medium overnight at 30 °C in a shake flask. The cells were then inoculated into 120 mL of SCD, $SCD^{complete}$ and $SCD^{leu,ile,val}$ or SCR, $SCR^{complete}$ and $SCR^{leu,ile,val}$ at an $OD_{600}$ (optical density measured at a wavelength of 600 nm) of 0.1, and were grown aerobically or anaerobically at 30 °C. For aerobic growth, the fermentation was performed in a shake flask. For anaerobic growth, the cells were grown without shaking in a flask that was sealed with a fermentation cap allowing $CO_2$ to escape without entry of oxygen. The $OD_{600}$ value was determined and gas-chromatography (GC) analysis performed after 6 h (time 1), 24 h (time 2), 30 h (time 3) and 48 h (time 4) of growth. All experiments were performed in triplicate.

To assess the effect of amino acid concentration on the formation of higher alcohols, a laboratory strain (BY4742) and a widely used commercial wine strain (VIN13) were precultured in 50 mL of SCD medium overnight at 30 °C. The cells were then inoculated into 120 mL $SCD^{leu}$, $SCD^{ile}$ and $SCD^{val}$ at an $OD_{600}$ of 0.1, and grown aerobically at 30 °C. The $OD_{600}$ value was determined and GC analysis performed.

## Recombinant DNA methods, and plasmid construction

Standard procedures for isolation of DNA were used throughout this study (Ausubel *et al.*, 1994). Restriction enzymes, T4 DNA ligase and Expand High-Fidelity DNA polymerase (Roche, Mannheim, Germany) were used in the enzymatic manipulation of DNA according to the specifications of the supplier.

The primers listed in Table 2 were used to amplify the coding regions of the various genes by means of the PCR technique. In order to identify possible cloning artifacts, all inserts were sequenced. The sequences obtained for the *BAT1* and *BAT2* genes of the commercial wine yeast strain, VIN13, were identical to those of the sequenced laboratory strain. Genomic DNA from VIN13 was used as the template to amplify the *BAT1* and *BAT2* coding sequences. A multicopy, episomal *S. cerevisiae – E. coli* shuttle vector pHVXII (Volschenk *et al.*, 1997) containing the promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences of the yeast phosphoglycerate kinase gene (*PGK1*) was used for subcloning the full-length *BAT1* and *BAT2* ORFs. PCR-generated DNA fragments (a 1182 bp *BAT1* fragment and a 1131-bp *BAT2* fragment) were digested with *Eco*RI and *Xho*I and subcloned into pHVXII, thereby generating pBAT1-m and pBAT2-m. The *Hin*dIII–*Hin*dIII fragments containing either the $PGK1_P$-*BAT1*-$PGK1_T$ or $PGK1_P$-*BAT2*-$PGK1_T$ gene cassettes were obtained from the respective multicopy plasmids and inserted into the unique *Hin*dIII site of plasmid pDLG42, generating single-copy integrating *S. cerevisiae – E. coli*

**Table 2.** Primers synthesized to amplify the genes

| Primer name | Sequence | Enzyme |
|---|---|---|
| BAT1′F | 5′-AATT**GAATTC**ATGTTGCAGAGACATTCC TTG-3′ | *Eco*RI |
| BAT1′R | 5′-TCGA**CTCGAG**TTAGTTCAAGTCGGCA ACAGT-3′ | *Xho*I |
| BAT2′F | 5′-AATT**GAATTC**ATGACCTTGGCACCCCT AGAC-3′ | *Eco*RI |
| BAT2′R | 5′-TCGA**CTCGAG**TCAGTTCAAATCAGTAA CAAC-3′ | *Xho*I |
| BAT1_probe_F | 5′-ATGTTGCAGAGACATTCCTTG-3′ | None |
| BAT1_probe_R | 5′-AAGCAGATGGGTCAAGAGAA-3′ | None |
| BAT2_probe_F | 5′-ATGACCTTGGCACCCCTAGA-3′ | None |
| BAT2_probe_R | 5′-TCAGTTCAAATCAGTAACAAC-3′ | None |
| ACT_probe_F | 5′-GACGCTCCTCGTGCTGTCTT-3′ | None |
| ACT_probe_R | 5′-GGAAGATGGAGCCAAAGCGG-3′ | None |

The enzyme sites are indicated in bold, and the regions homologous to the corresponding genes are underlined.

shuttle plasmids pBAT1-s and pBAT2-s. All plasmid inserts were sequenced and compared with the corresponding genomic sequence. Vector pDLG42 contained the dominant selectable *SMR1-410* marker gene (Casey *et al.*, 1988), a mutant allele of an endogenous gene of *S. cerevisiae* conferring resistance to the herbicide sulfometuron methyl, i.e. $Sm^R$. Plasmids pBAT1-s and pBAT2-s were linearized with *Apa*I in the *SMR1-410* terminator region for integration into the genome of the BY4742 laboratory strain and the VIN13 wine strain.

## Transformation

All bacterial transformations and isolation of DNA were carried out according to standard protocols (Sambrook *et al.*, 1989). Industrial wine yeast strains were transformed by means of electroporation. YPD (10 mL) was inoculated with yeast cells, and the cells incubated at 30 °C until reaching the stationary phase. A 500 mL volume of YPD was then inoculated with 10 mL of the yeast preculture and incubated until the mid-logarithmic growth phase was reached ($OD_{600}$ = 1.3–1.5). The *S. cerevisiae* cells were then harvested, washed with 80 mL of sterile water, re-suspended in 10 mL TE buffer (0.1 M Tris-HCl, 0.01 M EDTA, pH 7.5) (Sambrook *et al.*, 1989), and 10 mL of a 1 M lithium acetate stock solution. They were then incubated at 30 °C whilst being shaken gently. After 45 min, 2.5 mL of 1 M dithiothreitol (DTT) solution was added and there was further incubation for 15 min. The solution was then diluted to 500 mL with water and centrifuged. The cells were washed with 250 mL of ice-cold water and 30 mL of ice-cold 1 M sorbitol, and then suspended in 0.5 mL 1 M sorbitol; 40 μL of the concentrated yeast cells were added to 5–15 μg DNA in a sterile, ice-cold 1.5-mL tube. This was transferred to an ice-cold 0.4-cm gap electroporation cuvette. The Easyject T+450 V Twin pulse (EquiBio, Ashford, UK) apparatus was used for electroporation. The pulse program was as follows: voltage, 1500 V; capacity, 25 μF; shunt, 201 Ω. The yeast cells were then immediately plated out on SCDSM and incubated at 30 °C for at least 3 days.

## DNA and RNA extraction and Southern and northern blot analysis

Genomic DNA was isolated from yeast strains using the standard method (Ausubel *et al.*, 1994). The genomic DNA of VIN13(pBAT1-s) and BY4742(pBAT1-s) was digested with *Eco*RV, while the genomic DNA of VIN13(pBAT2-s) and BY4742(pBAT2-s) was digested with *Eco*RV and *Stu*I. The DNA fragments were separated by agarose gel electrophoresis and transferred to a Hybond-N nylon membrane (Amersham, Little Chalfont, UK).

For RNA extraction, Erlenmeyer flasks containing 100 mL of SCD (2% glucose) selective media were inoculated to an $OD_{600}$ of ∼0.1 from overnight precultures. The cultures were grown to an $OD_{600}$ of ∼1.0 and cells were harvested from 50 mL cell suspensions. Total RNA was extracted by the glass-bead mechanical disruption method (Ausubel *et al.*, 1994). *c.* 10 μg of total RNA was separated on 1.2% formaldehyde agarose gels. The RNA was transferred by capillary blot and UV-cross-linked to BioBond-Plus nylon membranes (Sigma-Aldrich, St Louis, MO).

Probes to detect the DNA or mRNA of the *BAT1*, *BAT2* and of the actin-encoding *ACT1* genes were generated through PCR with primers *BAT1*_probe_F, *BAT1*_probe_R, *BAT2*_probe_F, *BAT2*_probe_R, *ACT1*_probe_F and *ACT*_-probe_R (Table 2), and labelled using the PCR DIG probe synthesis kit (Roche Diagnostics). The labelled PCR products corresponded to the nucleotides +1 to +250 and +1 to +1131 of the *BAT1* and *BAT2* ORFs, respectively, and +73 and +972 of the *ACT1* ORF. After hybridization, the probe-target hybrids were visualized as described in the DIG application manual (Roche Diagnostics).

## Production and analysis of table wine

The wine yeast strains VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s) were each inoculated ($2 \times 10^6$ cells $mL^{-1}$) into 4.5 L of Colombard grape juice and fermented at 15 °C until dry (< 1 g $L^{-1}$ residual sugar). The wines were cold-stabilized, filtered and bottled according to standard practices for white wine production. All fermentations were done in triplicate and wine samples were scanned using a WineScan FT 120 instrument (Foss, Hillerød, Denmark) that employs a Michelson interferometer that was used to generate the FT-IR (Fourier infra-red) spectra. The samples (7 mL) were pumped through the $CaF_2$-lined cuvette (optical path-length 37 m) that is housed in the heater unit of

the instrument. The temperature of the samples was brought to exactly 40 °C before analysis. Samples were scanned from 5011 to 929 $cm^{-1}$ at 4 $cm^{-1}$ intervals, a range that includes a small section of the near infra-red (NIR) spectrum. The frequencies of the NIR beam transmitted by a sample were recorded at the detector and used to generate an interferogram. The latter was calculated from a total of 10 scans before being processed by Fourier transformation and corrected for the background absorbance of water to generate a single-beam transmittance spectrum. Two transmittance spectra for each sample were generated to calculate the absolute repeatability of the spectral measurements. The calculation of the absolute repeatability has been described in the laboratory manual of the supplier of the instrument (WineScan FT120 Type 77110 and 77310 reference Manual, Foss, Denmark, 2001). The transmittance spectra were finally converted into linearized absorbance spectra through a series of mathematical procedures.

## Base wine production and small-scale distillation

Wine yeast strains VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s) were inoculated ($2 \times 10^6$ cells $mL^{-1}$) into 15 L of Colombard grape juice, to which no sulphur dioxide was added, and fermented at 15 °C until dry. Fermentations were done in triplicate. Routine WineScan analysis was performed on the base wines just after alcoholic fermentation. Three 5-litre round-bottom flasks were each filled with 4.5 L of base wine and yeast lees derived from the original 15 L base wine fermentation volume. Two copper plates and 3 g of copper sulphate were added to the base wine and heated in heating mantles. The distillation flow rate was maintained at 5 mL $min^{-1}$, and the distillate was collected until 30% volume in volume(v/v) alcohol was reached. The same procedure was followed with the second distillation, except that the first 40 mL of distillate, collected at a flow rate of 2 mL $min^{-1}$, was discarded. The flow rate was then adjusted to 5 mL $min^{-1}$ and the heart was collected until 70% v/v alcohol was reached.

## Gas chromatography analysis

To each sample (10 mL of Colombard table or base wine), 0.8 mL of internal standard [230.2 mg $L^{-1}$ 4-methyl-2-pentanol and 12% (v/v) ethanol] and 6.5 mL of solvent (diethyl ether) were added. The tubes were then mechanically rotated at 60 rpm for 30 min. The top ether layer in each tube was separated and the extract analysed. For the 70% distillates, a 5-mL sample was taken, and 0.25 mL 4-methyl-2-pentanol [2 g $L^{-1}$ in 70% (v/v) ethanol stock solution] was added. After mixing, 2 μL of each sample was injected into the gas chromatograph (GC). The extractions were done in triplicate.

The analysis of volatile compounds was carried out on a Hewlett Packard 5890 Series II GC coupled to an HP 7673 auto-sampler and injector and an HP 3396A integrator. The column used was a Lab Alliance organic-coated, fused silica capillary with dimensions of 60 m × 0.32 mm internal diameter with a 0.5 μm coating thickness; hydrogen was used as the carrier gas for a flame ionisation detector held at 250 °C. The injector temperature was 200 °C; the split ratio 20 : 1; the flow rate 15 mL $min^{-1}$; and the injection volume 3 μL. The oven temperature program was as follows: 35 °C (10 min) to 230 °C (0 min) at a speed of 3 °C $min^{-1}$. For the distillate analysis, the conditions were as described above except for a different oven program and 2-μL injection volume: 30 °C (5 min) to 80 °C at a speed of 2 °C $min^{-1}$, and 80 °C (0 min) to 230 °C at a speed of 3 °C $min^{-1}$. For each of the compounds measured, a specific amount was measured for the standard used to calibrate the machine. The internal standard and the chemicals were sourced from Merck (Cape Town, South Africa).

Table wine extractions were performed after bottling. Extractions from the base wine were made after alcoholic fermentation and before distillation. Samples from the distillates were taken after the second distillation. The isoamyl alcohol and active amyl alcohol compounds could not be separated with the extraction and GC analyses; therefore the isoamyl alcohol concentrations throughout this paper also include the active amyl alcohol concentrations.

## Sensory evaluation

The table wines and distillates were sensorially evaluated for different fruity and flowery aromas, and for solvent or chemical intensity by a panel of six experienced judges. The wines and distillates were evaluated on a percentage scale from 0 to 100, where 0 represented the absence of a specific flavour and 100 represented a very high intensity of the flavour.

## Statistical analysis

For statistical analysis, the fermentation data obtained with the wild-type strains, BY4742 and VIN13, were regarded as control data sets and were always compared with the fermentation data obtained with the modified yeast strains, which were VIN13(pBAT1-s), VIN13(pBAT2-s), BY4742(-pBAT1-s), BY4742(pBAT2-s) and BY4742bat2Δ. The statistical differences between the GC results for the wines and the 70% distillates produced by the modified and the control yeast strains were determined using the Bonferroni test, in which the *P*-value is determined, and if $P \leq 0.05$ the difference is significant. The sensory evaluation data were statistically evaluated by determining the taste reliability and the *P*-values for the various aroma properties.

## Results

### Constitutive expression of BAT1 and BAT2 in a laboratory (BY4742) and an industrial (VIN13) yeast strain

In order to determine the effect of the yeast BCAATases on the concentration of higher alcohols and the flavour profile of wines and distillates, the *BAT1* and *BAT2* genes were cloned from the VIN13 wine yeast and placed under the regulatory sequences of the constitutively expressed *PGK1* gene. The $PGK1_P$-*BAT1*-$PGK1_T$ and $PGK1_P$-*BAT2*-$PGK1_T$ gene cassettes were inserted separately into a yeast-integrating plasmid to generate plasmids pBAT1-s and pBAT2-s. Linear copies of these *SMR1*-carrying plasmids were integrated into the genomic *ILV2* gene of the BY4742 laboratory strain and the VIN13 industrial strain, thereby generating transformants BY4742(pBAT1-s), BY4742(pBAT2-s), VIN13 (pBAT1-s) and VIN13(pBAT2-s). The integration of a single copy of either the $PGK1_P$-*BAT1*-$PGK1_T$ or $PGK1_P$-*BAT2*-$PGK1_T$ gene cassette in each of these transformants was confirmed by Southern blot analysis (Fig. 2). Northern blot analysis of the transformed strains confirmed that the integration resulted in a significant overexpression of *BAT1* and *BAT2* in the BY4742 background (Fig. 3). Similar results were obtained for the VIN13 strain (data not shown).

In addition to the strains in which *BAT1* or *BAT2* were overexpressed, BY4742 strains in which these two genes had been disrupted were also investigated. However, only the BY4742*bat2*Δstrain was available from EUROSCARF. Attempts to generate a BY4742*bat1*Δstrain resulted in a severe slow-growth phenotype (data not shown), and therefore only the effect of the disruption of *BAT2* and the overexpression of *BAT1* and *BAT2* on higher-alcohol production were assessed.

### Effect of the disruption of BAT2 and the overexpression of BAT1 and BAT2 on fermentation performance

The growth rate and final $OD_{600}$ of strains BY4742, BY4742(pBAT1-s), BY4742(pBAT2-s), BY4742*bat2*Δ, VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s) were determined under various growth conditions, including aerobic and anaerobic conditions with either glucose or raffinose as a carbon source, and in the presence of various amino acid concentrations. In addition, the industrial strains, VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s), were also assessed under winemaking conditions. No significant difference in either fermentation rate or final cell density was observed between the wild-type strains (BY4742 and VIN13) and their modified equivalents [BY4742(pBAT1-s), BY4742(-pBAT2-s), BY4742*bat2*Δ, VIN13(pBAT1-s) and VIN13(p-BAT2-s)] under any of the conditions tested. Neither was

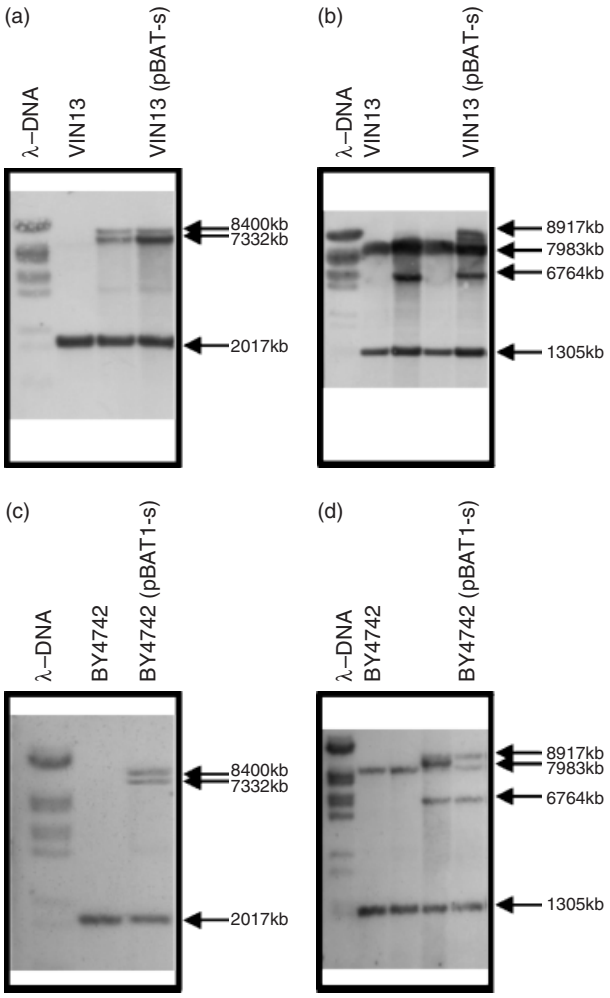


**Fig. 2.** Genomic DNA analysis of *BAT1* and *BAT2*. The first lanes were loaded with *Bst*EII-digested lambda DNA. The other lanes contain genomic DNA digested by *Eco*RV (panels a and c) and by *Eco*RV and *Stu*I (panels b and d). The panels show the signals of the wild-type copies of *BAT1* and *BAT2* and of the integrated copies of the genes in the corresponding strains. All bands are of the expected size as indicated on the panels.

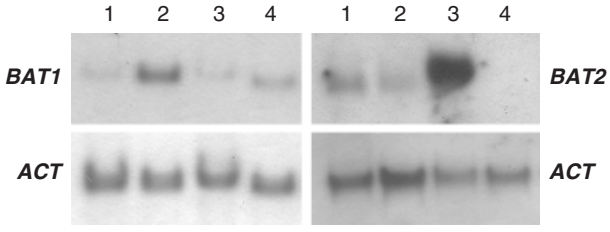


**Fig. 3.** Transcriptional regulation of *BAT1* and *BAT2* in the BY4742 yeast strain. The top panel indicates the mRNA levels of *BAT1* (left) and *BAT2* (right), while the bottom panels represent the *ACT* (actin) expression levels in both cases. The lanes are as follows. 1: BY4742 (wild type), 2: BY4742(pBAT1-s), 3: BY4742(pBAT2-s) and 4: By4742bat2Δ.

**Table 3.** Analysis of the Colombard white wine after bottling and of the Colombard base wine after alcoholic fermentation and before distillation

| | Colombard white wine after bottling | | | Colombard base wine after fermentation, just before distillation | | |
|---|---|---|---|---|---|---|
| Parameters | VIN13 | VIN13 (pBAT1-s) | VIN13 (pBAT2-s) | VIN13 | VIN13 (pBAT1-s) | VIN13 (pBAT2-s) |
| pH | 3.42 | 3.44 | 3.43 | 3.74 | 3.70 | 3.77 |
| Volatile acid ($g\,L^{-1}$) | 0.26 | 0.28 | 0.25 | 0.32 | 0.23 | 0.28 |
| Total acids ($g\,L^{-1}$) | 5.38 | 5.41 | 5.35 | 5.20 | 5.48 | 5.27 |
| Malic acid ($g\,L^{-1}$) | 2.82 | 2.92 | 2.86 | 2.99 | 3.47 | 3.21 |
| Lactic acid ($g\,L^{-1}$) | 0.18 | 0.13 | 0.16 | 0.07 | 0.13 | 0.02 |
| Glucose ($g\,L^{-1}$) | 0.16 | 0.07 | 0.06 | 0.06 | 0.48 | 0.10 |
| Fructose ($g\,L^{-1}$) | 2.08 | 2.80 | 2.35 | 1.27 | 0.55 | 0.94 |
| Glycerol ($g\,L^{-1}$) | 5.48 | 5.64 | 5.40 | 5.28 | 5.28 | 5.10 |
| Ethanol (%v/v) | 11.49 | 11.68 | 11.58 | 11.96 | 12.22 | 12.63 |

there any significant difference in the concentrations of ethanol, glycerol, lactic acid, malic acid, volatile acids, and total acids in the table and base wines produced with the modified VIN13 strains (Table 3). It therefore appears that neither the disruption of *BAT2* nor the overexpression of *BAT1* and *BAT2* has any obvious negative impact on the general growth and fermentation performances of the strains.

## Effect of the disruption of BAT2 and the overexpression of BAT1 and BAT2 on the accumulation of higher alcohols

The laboratory and industrial strains in which the *BAT1* and *BAT2* genes had been modified were tested under various culture conditions, namely (i) in glucose- and raffinose-containing media; (ii) with and without various additional amino acids; and (iii) under aerobic and anaerobic conditions. The data for each of the conditions are presented in Tables 4–7.

The data presented in Table 4 suggest that isobutanol production by the laboratory strain BY4742 is not affected significantly by the growth conditions, since similar concentrations were found whether the strain had been grown aerobically or anaerobically, or with glucose or raffinose as sole carbon source. Compared with this strain, a 2-fold reduction in isobutanol production was observed when the BY4742*bat2*Δdeletion strain was used in all the tested conditions, suggesting that Bat2p-dependent activity contributes significantly to isobutanol production. The contribution of *BAT2* appears to be unaffected by glucose concentration or oxygen availability.

The overexpression of *BAT2* in BY4742 resulted in significant increases in isobutanol concentrations (Table 4), further supporting the role that *BAT2* plays in the accumulation of this metabolite in *Saccharomyces cerevisiae*. The overexpression of *BAT1* also resulted in increased levels of isobutanol concentrations, but the total amounts were significantly lower than in the case of *BAT2* overexpression. Contrary to the case for the BY4742 reference laboratory strain, the levels of isobutanol produced were considerably higher when the *BAT1* and *BAT2* overexpression strains were grown anaerobically. The isobutanol concentrations increased 3.6-fold and 1.4-fold in BY4742(pBAT2-s) and BY4742(pBAT1-s), respectively, when grown aerobically, and 10.8-fold and 3-fold when grown in anaerobic conditions. While the transformed VIN13 strains [VIN13(pBAT1-s) and VIN13(pBAT2-s)] followed a similar trend to the laboratory transformants [BY4742(pBAT2-s) and BY4742 (pBAT1-s)] regarding the effect of the constitutive expression of *BAT1* and *BAT2* on isobutanol production, the strains produced two times less isobutanol when grown anaerobically compared with when they were grown aerobically.

When increased levels of valine, the direct precursor of isobutanol, were added to the media at two different concentrations, significant increases in isobutanol concentrations were observed in all the strains and in most conditions used (Table 4). The only exception was when the strains were grown anaerobically in a raffinose medium; the isobutanol concentrations remained approximately the same for all the strains whether grown in the presence or absence of valine. The relative contributions of the *BAT1* and *BAT2* genes to the increases in isobutanol production were unaffected by the addition of valine. In the same growth conditions, VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s) continued to produce 2-fold lower isobutanol concentrations when the strains were grown anaerobically in glucose or raffinose compared with when grown aerobically. Interestingly, in these conditions, BY4742 and BY4742*bat2*Δ-showed a similar tendency (Figs 4 and 5).

The isobutyric acid concentrations (Table 5) were affected in similar ways to the isobutanol concentrations by the various strains in all the conditions tested. In this case, however, the concentrations were significantly lower when the strains were grown anaerobically. This is probably a

**Table 4.** Isobutanol concentrations (mg $L^{-1}$) produced by the various strains in different growth conditions

| | Glucose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Raffinose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Glucose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Raffinose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Glucose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isoleucine) | | Raffinose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isoleucine) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yeast strain | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic |
| VIN13 | 12.63 ± 1.35 | 4.68 ± 0.43 | 6.70 ± 1.02 | 3.78 ± 0.03 | 13.49 ± 1.06 | 6.65 ± 0.88 | 6.66 ± 0.48 | 5.19 ± 0.15 | 14.51 ± 2.29 | 8.38 ± 2.38 | 7.36 ± 0.16 | 4.96 ± 0.39 |
| VIN13(pBAT1-s) | 44.27 ± 3.66 | 25.77 ± 2.66 | 58.37 ± 0.08 | 28.60 ± 0.40 | 49.49 ± 2.33 | 25.93 ± 3.25 | 36.34 ± 2.26 | 23.81 ± 0.20 | 50.01 ± 5.13 | 33.91 ± 3.62 | 40.47 ± 2.65 | 27.06 ± 1.48 |
| VIN13(pBAT2-s) | 134.51 ± 14.04 | 50.57 ± 4.26 | 65.59 ± 4.35 | 34.07 ± 0.01 | 141.42 ± 22.11 | 84.9 ± 7.49 | 62.39 ± 1.45 | 43.56 ± 0.16 | 134.58 ± 12.05 | 90.36 ± 8.33 | 60.87 ± 2.83 | 44.05 ± 3.93 |
| BY4742 | 11.13 ± 0.98 | 9.86 ± 0.97 | 8.62 ± 0.44 | 9.69 ± 0.70 | 47.85 ± 0.41 | 34.83 ± 2.09 | 19.01 ± 0.18 | 12.59 ± 1.57 | 26.38 ± 2.59 | 19.25 ± 0.20 | 14.29 ± 1.11 | 6.97 ± 0.35 |
| BY4742(pBAT1-s) | 16.08 ± 1.93 | 29.41 ± 4.43 | 13.45 ± 0.09 | 24.36 ± 3.24 | 57.23 ± 1.61 | 72.06 ± 10.80 | 29.78 ± 0.58 | 30.22 ± 1.74 | 38.32 ± 2.25 | 56.13 ± 7.38 | 28.13 ± 0.47 | 24.93 ± 5.04 |
| BY4742(pBAT2-s) | 40.60 ± 1.63 | 106.63 ± 4.16 | 29.02 ± 1.32 | 48.44 ± 1.62 | 75.99 ± 1.31 | 149.00 ± 19.51 | 64.54 ± 1.48 | 40.57 ± 0.81 | 73.70 ± 4.00 | 126.87 ± 4.89 | 68.1 ± 3.63 | 36.48 ± 0.16 |
| BY4742Δbat2 | 6.09 ± 0.96 | 5.64 ± 1.41 | 4.92 ± 0.17 | 4.57 ± 0.63 | 36.28 ± 1.42 | 20.43 ± 1.84 | 12.24 ± 0.55 | 7.77 ± 1.06 | 14.03 ± 0.80 | 10.51 ± 0.63 | 8.32 ± 0.23 | 3.41 ± 0.36 |

**Table 5.** Isobutyric acid concentration (mg $L^{-1}$) produced by the various strains in different growth conditions

| | Glucose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Raffinose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Glucose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Raffinose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Glucose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isoleucine) | | Raffinose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isoleucine) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yeast strain | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic |
| VIN13 | 2.51 ± 0.42 | 0.61 ± 0.16 | 2.71 ± 0.16 | 0.50 ± 0.01 | 3.09 ± 0.37 | 0.87 ± 0.61 | 2.31 ± 0.02 | 0.87 ± 0.02 | 3.57 ± 0.96 | 0.76 ± 0.15 | 2.80 ± 0.00 | 0.83 ± 1.49 |
| VIN13(pBAT1-s) | 10.27 ± 1.37 | 2.74 ± 0.23 | 23.65 ± 0.17 | 3.52 ± 0.15 | 13.07 ± 0.91 | 2.43 ± 0.37 | 13.7 ± 1.34 | 4.18 ± 0.16 | 13.23 ± 1.66 | 3.52 ± 0.19 | 17.22 ± 1.89 | 5.56 ± 0.18 |
| VIN13(pBAT2-s) | 33.71 ± 5.77 | 5.44 ± 0.42 | 28.82 ± 1.40 | 6.24 ± 0.18 | 44.69 ± 4.94 | 8.61 ± 0.29 | 30.32 ± 1.52 | 9.53 ± 0.63 | 44.82 ± 5.86 | 9.32 ± 1.25 | 32.12 ± 0.15 | 9.7 ± 0.09 |
| BY4742 | 2.43 ± 0.71 | 0.99 ± 0.21 | 2.67 ± 0.53 | 1.34 ± 0.29 | 6.51 ± 0.62 | 1.93 ± 0.05 | 2.82 ± 0.05 | 1.72 ± 0.17 | 4.10 ± 0.15 | 1.48 ± 0.06 | 2.37 ± 0.15 | 1.3 ± 0.18 |
| BY4742(pBAT1-s) | 2.66 ± 0.38 | 1.81 ± 0.31 | 4 ± 0.19 | 3.46 ± 0.45 | 7.31 ± 0.34 | 3.71 ± 0.28 | 4.46 ± 0.16 | 3.59 ± 0.04 | 5.58 ± 0.20 | 3.80 ± 0.71 | 4.48 ± 0.01 | 3.72 ± 0.65 |
| BY4742(pBAT2-s) | 9.23 ± 0.32 | 8.46 ± 0.34 | 8.73 ± 0.84 | 10.17 ± 0.27 | 14.20 ± 1.55 | 11.75 ± 0.97 | 12.71 ± 1.27 | 8.04 ± 0.14 | 17.22 ± 1.19 | 12.50 ± 0.11 | 13.70 ± 1.50 | 8.88 ± 0.28 |
| BY4742Δbat2 | 0.97 ± 0.30 | 0.55 ± 0.17 | 1.3 ± 0.05 | 0.58 ± 0.06 | 3.73 ± 0.23 | 0.99 ± 0.12 | 1.58 ± 0.02 | 0.75 ± 0 | 1.99 ± 0.11 | 0.72 ± 0.13 | 1.40 ± 0.04 | 0.57 ± 0.12 |

**Table 6.** Isoamyl alcohol concentration (mg $L^{-1}$) produced by the various strains in different growth conditions

| | Glucose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Raffinose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Glucose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Raffinose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Glucose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isolecine) | | Raffinose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isolecine) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yeast strain | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic |
| VIN13 | 42.42 ± 1.81 | 41.54 ± 0.40 | 28.43 ± 2.04 | 30.83 ± 0.15 | 22.12 ± 0.67 | 24.25 ± 1.74 | 11.87 ± 0.49 | 12.85 ± 0.06 | 35.91 ± 3.22 | 28.98 ± 3.32 | 24.53 ± 2.59 | 16.19 ± 1.04 |
| VIN13(pBAT1-s) | 57.22 ± 1.60 | 69.29 ± 3.99 | 39.25 ± 0.13 | 39.41 ± 0.13 | 41.8 ± 1.16 | 50.26 ± 4.26 | 23.26 ± 1.46 | 22.23 ± 0.45 | 40.09 ± 1.78 | 38.85 ± 2.04 | 27.98 ± 2.18 | 22.7 ± 0.68 |
| VIN13(pBAT2-s) | 62.09 ± 2.07 | 55.07 ± 1.96 | 34.36 ± 0.59 | 34.33 ± 0.40 | 44.16 ± 3.27 | 47.12 ± 3.02 | 24.56 ± 1.03 | 20.62 ± 0.77 | 73.79 ± 3.73 | 65.47 ± 3.16 | 42.99 ± 1.70 | 28.77 ± 1.36 |
| BY4742 | 27.78 ± 2.04 | 29.31 ± 0.93 | 27.88 ± 0.33 | 28.6 ± 0.96 | 13.28 ± 0.55 | 13.37 ± 2.21 | 16.24 ± 0.14 | 7.45 ± 0.25 | 22.39 ± 1.31 | 17.68 ± 0.11 | 29.98 ± 0.45 | 11.9 ± 0.79 |
| BY4742(pBAT1-s) | 27.52 ± 2.43 | 29.11 ± 0.68 | 27.27 ± 0.33 | 29.62 ± 1.56 | 12.43 ± 0.60 | 11.42 ± 0.46 | 14.28 ± 0.23 | 6.97 ± 0.07 | 21.28 ± 0.91 | 16.05 ± 0.59 | 29.21 ± 0.78 | 11.61 ± 0.64 |
| BY4742(pBAT2-s) | 32.65 ± 1.82 | 38.68 ± 1.17 | 31.37 ± 0.09 | 31.65 ± 0.95 | 18.10 ± 1.05 | 21.34 ± 2.97 | 24.5 ± 2.43 | 10.41 ± 0.63 | 36.82 ± 0.77 | 29.74 ± 0.35 | 51.67 ± 3.42 | 18.45 ± 0.86 |
| BY4742Δbat2 | 26.17 ± 2.57 | 28.25 ± 2.02 | 27.04 ± 0.03 | 27.24 ± 1.59 | 9.89 ± 0.64 | 9.2 ± 0.12 | 12.77 ± 0.05 | 6.05 ± 0.64 | 15.28 ± 0.50 | 16.75 ± 3.35 | 21.81 ± 1.58 | 9.7 ± 0.46 |

**Table 7.** Isovaleric acid concentration (mg $L^{-1}$) produced by the various strains in different growth conditions

| | Glucose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Raffinose (without valine or isoleucine addition; 59.76 mg $L^{-1}$ leucine) | | Glucose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Raffinose (125.94 mg $L^{-1}$ valine; 50.38 mg $L^{-1}$ leucine; 34.98 mg $L^{-1}$ isoleucine) | | Glucose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isoleucine) | | Raffinose (225.94 mg $L^{-1}$ valine; 150.38 mg $L^{-1}$ leucine; 134.98 mg $L^{-1}$ isoleucine) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yeast strain | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic |
| VIN13 | 1.26 ± 0.07 | 0.74 ± 0.04 | 2.74 ± 0.36 | 1.18 ± 0.03 | 0.71 ± 0.01 | 0.34 ± 0.01 | 0.87 ± 0.04 | 0.45 ± 0.05 | 1.67 ± 0.14 | 0.42 ± 0.08 | 2.03 ± 0.10 | 0.57 ± 0.02 |
| VIN13(pBAT1-s) | 1.94 ± 0.18 | 1.07 ± 0.06 | 4.06 ± 0.04 | 1.64 ± 0.12 | 1.38 ± 0.12 | 0.7 ± 0.05 | 1.58 ± 0.3 | 0.83 ± 0.08 | 1.57 ± 0.30 | 0.63 ± 0.01 | 2.46 ± 0.43 | 0.92 ± 0.09 |
| VIN13(pBAT2-s) | 4.11 ± 0.50 | 2.92 ± 0.24 | 4.91 ± 0.37 | 5.39 ± 0.05 | 2.24 ± 0.18 | 0.92 ± 0.09 | 2.54 ± 0.03 | 1.11 ± 0.22 | 3.93 ± 0.48 | 1.33 ± 0.10 | 4.59 ± 0.10 | 1.47 ± 0.14 |
| BY4742 | 1.41 ± 0.34 | 0.92 ± 0.10 | 1.83 ± 0.19 | 2 ± 0.05 | 0.48 ± 0.06 | 0.28 ± 0.06 | 0.54 ± 0.08 | 0.35 ± 0.05 | 0.66 ± 0.06 | 0.38 ± 0.01 | 0.85 ± 0.04 | 0.5 ± 0.02 |
| BY4742(pBAT1-s) | 1.36 ± 0.23 | 0.94 ± 0.10 | 1.84 ± 0.46 | 2.07 ± 0.11 | 0.28 ± 0.25 | 0.27 ± 0.04 | 0.48 ± 0.09 | 0.26 ± 0.01 | 0.62 ± 0.07 | 0.41 ± 0.12 | 0.76 ± 0.04 | 0.47 ± 0.01 |
| BY4742(pBAT2-s) | 2.51 ± 0.36 | 2.06 ± 0.12 | 2.96 ± 0.82 | 4.26 ± 0.70 | 0.82 ± 0.08 | 0.65 ± 0.10 | 1.05 ± 0.13 | 0.66 ± 0.01 | 1.42 ± 0.19 | 0.93 ± 0.02 | 1.74 ± 0.15 | 1.03 ± 0.05 |
| BY4742Äbat2 | 1.15 ± 0.21 | 0.84 ± 0.02 | 1.78 ± 0.11 | 1.93 ± 0.13 | 0.37 ± 0.07 | 0.20 ± 0.04 | 0.43 ± 0.04 | 0.3 ± 0.01 | 0.46 ± 0.06 | 0.32 ± 0.05 | 0.62 ± 0.02 | 0.41 ± 0.00 |

result of the fact that the biosynthesis is an oxidative process, which requires $NAD^+$.

There were no significant differences in isoamyl alcohol concentrations produced by the BY4742 wild-type, overexpression [BY4742(pBAT2-s) and BY4742(pBAT1-s)] or deletion (BY4742*bat2*Δ) strains when grown in glucose or raffinose media, with or without oxygen, (Table 6). However, a 1.5-fold decrease in isoamyl alcohol concentrations was observed for the VIN13 wild-type and overexpression strains [VIN13(pBAT1-s) and VIN13(pBAT2-s)] when grown in raffinose compared with a glucose medium. VIN13(pBAT1-s) showed a 1.4-fold increase in isoamyl alcohol concentration in all conditions compared with VIN13, and VIN13(pBAT2-s) and BY4742(pBAT2-s) showed a 1.3-fold increase in isoamyl alcohol concentration. VIN13(pBAT1-s) also produced higher isoamyl alcohol concentrations than VIN13(pBAT2-s) when grown anaerobically. The same tendencies were observed when different leucine and isoleucine concentrations were assessed.

The isovaleric acid concentrations remained approximately the same for the wild-type and *BAT1* overexpression strains in all the culture conditions. In the strains in which the *BAT2* gene was overexpressed and in all of the growth conditions, a 2- to 3-fold increase in isovaleric acid was detected (Table 7). With the BY4742*bat2*Δ strain, a 1.3-fold decrease in isovaleric acid concentration was measured in all of the conditions.

To assess the effect of amino acid concentrations on the formation of higher alcohols, the BY4742 laboratory strain and the VIN13 industrial strain (data not shown) were grown aerobically in a minimal synthetic medium containing high concentrations of leucine, isoleucine or valine, respectively. The growth and the concentration of the aroma compounds were evaluated over a 54 h period (data not

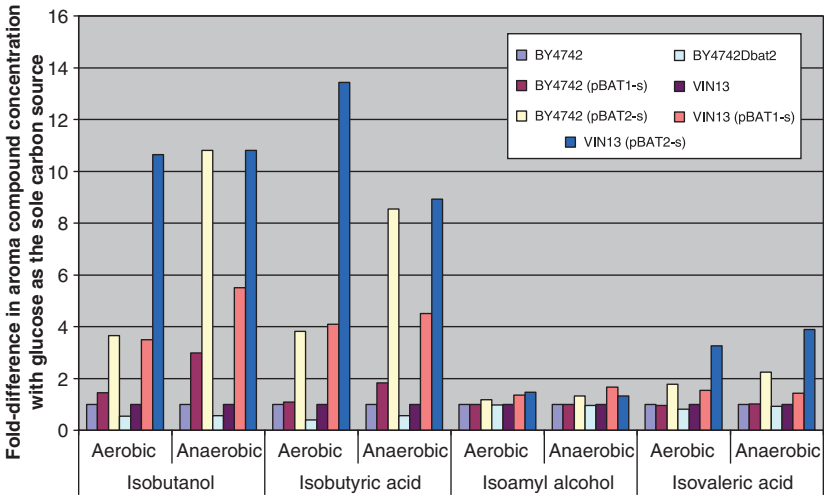


**Fig. 4.** The fold-difference in aroma compound concentration (mg $L^{-1}$) produced by the different strains with glucose as the sole carbon source.

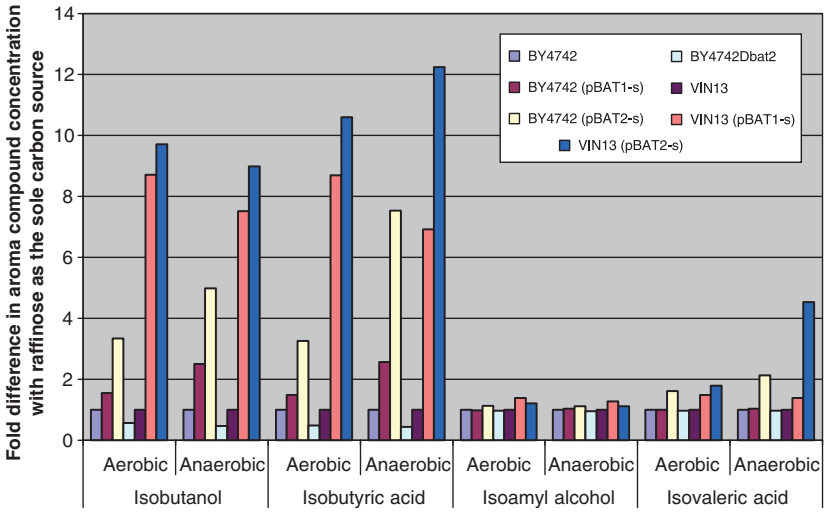


**Fig. 5.** The fold-difference in aroma compound concentration (mg $L^{-1}$) produced by the different strains with raffinose as the sole carbon source.

shown). Both yeast strains reached the stationary phase after 24 h. Interestingly, the two most important higher alcohols, isoamyl alcohol and propanol, as well as isobutyric acid and isovaleric acid also reached maximum concentrations after 24 h. These concentrations remained constant for the remainder of the time. However, the concentrations of isobutanol and 2-phenylethyl alcohol increased throughout the monitored period, indicating continued production during the stationary phase.

After 54 h of growth, the synthetic media in which the control and modified strains of BY4742 and VIN13 strains were grown were analysed using gas chromatography (Figs 6 and 7). The data showed that both strains reacted similarly when an additional 100 mg of the amino acids valine, leucine and isoleucine were individually added to the synthetic media. The addition of valine to the media caused statistically significant increases in isobutanol, isobutyric acid, propanol, and propionic acid concentrations when compared with standard synthetic media without the added amino acid. When leucine was added to the media, the isoamyl alcohol concentrations increased 3.4- and 3.1-fold during growth for VIN13 and BY4742, respectively. The addition of isoleucine to the synthetic media also resulted in 2.2- and 1.8-fold increases in isoamyl alcohol concentrations compared with the media with added valine. The addition of high concentrations of leucine to the media also caused 1.3- and 2-fold increases in 2-phenylethyl alcohol concentrations in the VIN13 and BY4742 culture media, respectively. The addition of leucine and isoleucine to the synthetic media caused 3.8- and 3-fold increases in isovaleric acid concentrations compared with the media with added valine. The concentrations of the other compounds evaluated did not show any significant differences.

## Effect of the disruption of BAT2 and the overexpression of BAT1 and BAT2 on the accumulation of other aroma compounds

A metabolic map that links all metabolites listed in Table 9 to the BCAA metabolism was developed to analyse the effects of the modification of *BAT1* and *BAT2* expression further.

While the data described in the previous section clearly show that both *BAT1* and *BAT2* play a direct role in the

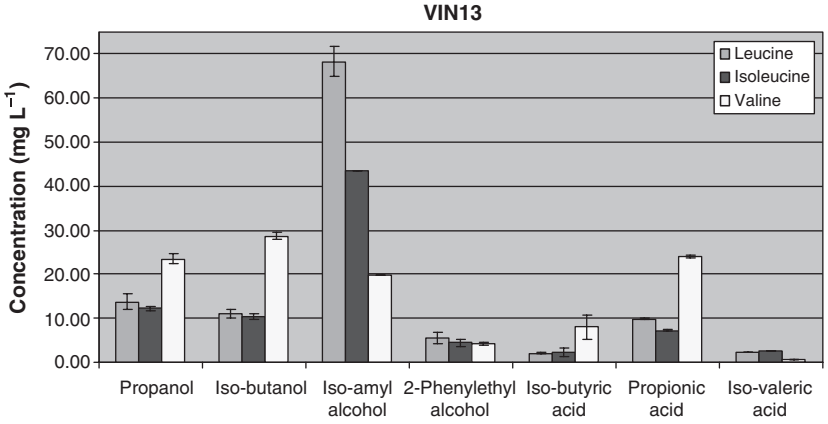


**Fig. 6.** Higher alcohols and fatty acids produced by VIN13 when specific amino acids are added to the fermentation media.

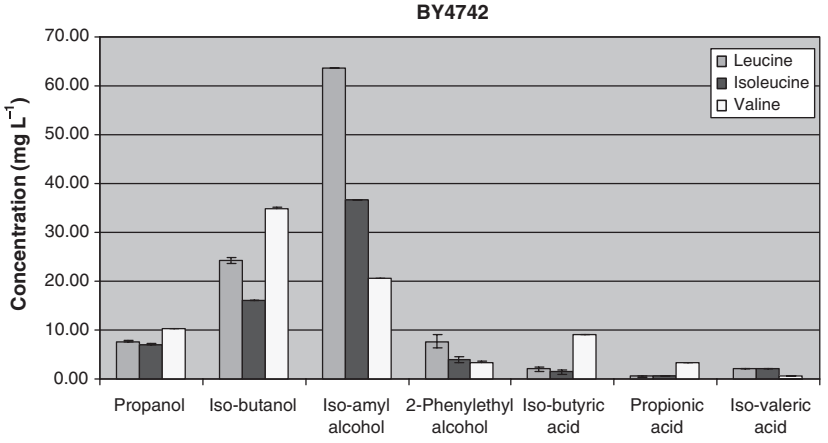


**Fig. 7.** Higher alcohols and fatty acids produced by BY4742 when specific amino acids are added to the fermentation media.

formation of higher alcohols, they also indicate that other aroma-active compounds, some of which appear not to be linked directly to the higher-alcohol metabolism, are affected by the changes in *BAT* gene expression. In particular, significant effects on the production of fatty acids were observed, specifically hexanoic, octanoic and decanoic acids. However, the concentrations of these fatty acids varied quite significantly throughout the experimental time-frame and for the different growth conditions (data not shown). For example, while hexanoic acid appeared to be generally unaffected by the changes in either *BAT1* or *BAT2* expression, large increases in concentrations were observed at the end of aerobic growth of the BY4742(pBAT1-s) strain in some growth conditions. Octanoic acid production was significantly reduced at the start of the aerobic growth of the BY4742(pBAT1-s) strain in media containing added amino acids and significantly increased when the same strain was grown anaerobically. A similar pattern was observed for aerobic and anaerobic growth of the BY4742(-pBAT2-s) and the BY4742(*bat2*Δ) strains when grown in the same medium.

The above fatty acids can be further metabolized into important aroma compounds by the formation of ethyl esters. The formation of ethyl caproate, ethyl caprylate and ethyl caprate was monitored during this study to determine if *BAT1* or *BAT2* had any effect on their production. However, few consequences were observed on the production of these compounds.

## Effect of the overexpression of BAT1 and BAT2 on aroma compound concentrations in wine

Concentrations of certain esters, higher alcohols and acids important to wine aroma were determined for the Colombard table and base wines and the corresponding distillates (Table 8). The statistical significance of the data (*P*-values) is

**Table 8.** The effect of yeast strain on the concentration of major volatiles in Colombard white wines, Colombard base wines, and the respective 70% distillates

| | Concentration (mg $L^{-1}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Colombar white wine after bottling | | | Colombar base wine after alcoholic fermentation | | | 70% distillate after second distillation | | |
| Component | VIN13 | VIN13 (pBAT1-s) | VIN13 (pBAT2-s) | VIN13 | VIN13 (pBAT1-s) | VIN13 (pBAT2-s) | VIN13 | VIN13 (pBAT1-s) | VIN13 (pBAT2-s) |
| Acetic acid | 109.62 | 68.91 | 129.62 | 212.29 | 149.63 | 141.81 | 14.82 | **1.27** | **3.56** |
| Decanoic acid | 3.18 | 2.44 | 4.02 | 5.37 | 6.81 | 6.33 | 41.98 | 45.55 | 45.97 |
| Hexanoic acid | 6.40 | **4.96** | **8.35** | 7.55 | 8.43 | 8.08 | 34.35 | 48.56 | 48.15 |
| *i*-Butyric acid | 1.06 | 0.90 | **5.66** | 0.62 | **1.06** | **3.58** | 1.00 | 1.35 | **5.09** |
| *n*-Butyric acid | 0.69 | 0.52 | 0.82 | 1.77 | 1.71 | 1.72 | 1.48 | 1.09 | 0.70 |
| *i*-Valeric acid | 0.69 | 0.66 | **1.19** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *n*-Valeric acid | 0.53 | **0.23** | 0.49 | 0.70 | 0.51 | 0.57 | 0.00 | 0.00 | 0.00 |
| Octanoic aicd | 9.63 | 7.27 | 12.46 | 11.40 | 12.96 | 12.42 | 49.54 | 48.06 | 52.38 |
| Propionic acid | 0.79 | **2.18** | **6.31** | 31.01 | 27.24 | 32.72 | 0.00 | 0.00 | 0.00 |
| Acetate | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 89.54 | 69.58 | 70.44 |
| 2-Phenylethyl acetate | 0.39 | **0.29** | 0.44 | 0.55 | 0.51 | 0.48 | 2.66 | 2.53 | 2.57 |
| Diethyl succinate | 0.16 | 0.22 | **0.48** | 1.14 | **1.44** | **1.45** | 2.55 | 2.79 | **3.31** |
| Ethyl acetate | 71.77 | **52.38** | 83.75 | 95.54 | 89.32 | 88.14 | 250.10 | 222.19 | 234.39 |
| Ethyl butyrate | 0.00 | 0.00 | 0.47 | 0.31 | 0.53 | 0.53 | 0.70 | 0.37 | 1.05 |
| Ethyl caproate C6 | 1.86 | **1.40** | 2.19 | 2.14 | 1.99 | 2.02 | 8.96 | 8.02 | 8.95 |
| Ethyl caprylate C8 | 1.63 | 1.24 | 2.03 | 2.11 | 2.18 | 2.16 | 13.71 | 15.43 | 16.47 |
| Ethyl caprate C10 | 1.60 | **1.02** | 1.63 | 1.42 | 1.39 | 1.41 | 29.63 | 36.96 | 36.93 |
| Ethyl lactate | 1.34 | 1.00 | **2.15** | 0.00 | 0.16 | 0.20 | 2.25 | 2.35 | **3.08** |
| Hexyl acetate | 1.17 | **0.89** | 1.40 | 1.35 | 1.24 | 1.17 | 6.51 | 5.67 | 5.74 |
| Iso-amyl acetate | 8.52 | **10.13** | **6.83** | 10.01 | **14.52** | **7.13** | 39.17 | 51.41 | 27.93 |
| Acetoin | 0.63 | 0.91 | 2.01 | 0.00 | 0.00 | 0.16 | 0.00 | 0.00 | 0.00 |
| Methanol | 24.57 | 19.48 | **34.20** | 43.41 | 37.94 | 39.95 | 216.87 | 209.57 | 218.71 |
| 2-Phenylethyl alcohol | 9.62 | **6.60** | **10.62** | 13.67 | 12.17 | **11.80** | 8.50 | 6.98 | 7.44 |
| Propanol | 32.88 | **18.14** | 36.80 | 41.66 | **32.73** | 41.23 | 256.69 | 215.24 | 268.22 |
| Iso-butanol | 5.62 | **11.33** | **81.63** | 8.87 | **15.06** | **85.85** | 61.60 | **111.00** | **607.84** |
| *n*-Butanol | 0.98 | **0.22** | **0.48** | 1.15 | **0.38** | **0.48** | 6.56 | **2.36** | **2.73** |
| Iso-amyl alcohol | 129.57 | **177.92** | 106.71 | 121.54 | **163.09** | 115.95 | 716.02 | **1008.68** | 701.63 |
| Hexanol | 1.62 | **1.14** | **1.97** | 2.07 | 1.96 | 2.05 | 12.34 | 12.21 | 12.42 |

Values in bold differ significantly from VIN13 on a 5% significance level.

presented in Table 9. The table wine fermented with VIN13(pBAT1-s) showed an overall decrease in ester concentrations compared with the control wine. The concentrations of 2-phenylethyl acetate, ethyl caprylate and hexyl acetate decreased 1.3-fold, ethyl acetate and ethyl caproate decreased 1.4-fold, and ethyl caprate decreased 1.6-fold compared with the concentrations in the table wine fermented with VIN13. However, a 1.2-fold increase in isoamyl acetate concentration was obtained in the table wine fermented with VIN13(pBAT1-s) compared with the control wine. The table wine produced with VIN13(pBAT2-s) showed a slight increase in total ester concentration. Diethyl succinate concentration increased 3-fold, ethyl acetate and hexyl acetate concentrations increased 1.2-fold, and the concentration of ethyl lactate increased 1.6-fold in the wines fermented with VIN13(pBAT2-s). However, the isoamyl acetate concentration of the VIN13(pBAT2-s)-fermented table wine decreased 1.2-fold. The ester concentrations of the base wines did not differ significantly except for a 1.3-fold increase in diethyl succinate concentration in both the wines fermented with VIN13(pBAT1-s) and VIN13(pBAT2-s) compared with the VIN13-fermented base wine. A considerable increase in isoamyl acetate concentration in the VIN13(pBAT1-s)-fermented base wine and a 1.4-fold decrease in isoamyl acetate concentration in the base wine fermented with VIN13(pBAT2-s) were also observed. These results correlated with the corresponding distillates.

The table wines, base wines and distillates produced with VIN13(pBAT2-s) contained the highest fusel alcohol concentrations compared with the products produced with VIN13(pBAT1-s), which in turn had higher fusel alcohol concentrations than the control wines and distillates (Table 8). The table wine fermented with VIN13(pBAT1-s) showed a 1.5-fold decrease in 2-phenylethyl alcohol concentration, and 1.4-fold, 1.8-fold and 4.5-fold decreases in hexanol, propanol and *n*-butanol concentrations, respectively. These wines also presented 2-fold increases in isobutanol concentrations and 1.4-fold increases in isoamyl alcohol concentrations. Increases in propanol, 2-phenylethyl alcohol and hexanol concentrations, as well as a 14.5-fold increase in isobutanol concentration, were observed in the VIN13(pBAT2-s)-fermented table wine compared with the control wines. 3- and 2.4-fold decreases in *n*-butanol concentrations and 1.7- and 9.7-fold increases in isobutanol concentrations were detected in the base wines fermented with VIN13(pBAT1-s) and VIN13(pBAT2-s), respectively. 1.4-fold increases in isoamyl alcohol concentrations were measured in the base wines fermented with VIN13(pBAT1-s), and decreases in isoamyl alcohol concentrations were measured in the VIN13(pBAT2-s)-fermented base wines. Decreases in 2-phenylethyl alcohol concentrations were observed in both the wines fermented with the modified strains compared with the control. 1.3-fold decreases in propanol concentrations were measured in the VIN13(pBAT1-s)-fermented base wines, and propanol concentrations increased in the VIN13(pBAT2-s)-fermented base wines. These results correlated with the data from the distillates of the corresponding base wines.

There were overall decreases in acetic acid concentrations in the wines and distillates produced with the transformed wine yeast strains (Table 8). The isobutyric acid concentrations increased between 1.2- and 1.7-fold in the products produced with VIN13(pBAT1-s), and between 5.1- and 5.8-fold in the VIN13(pBAT2-s)-derived products compared with those products produced with VIN13. The table wines fermented with VIN13(pBAT2-s) had 1.7-fold increases in isovaleric acid and 8-fold increases in propionic acid concentrations. The VIN13(pBAT1-s)-fermented table wines showed 2.3-fold decreases in *n*-valeric acid concentrations and 2.8-fold increases in propionic acid concentrations. Isovaleric acid, *n*-valeric acid and propionic acid were not detected in the distillates.

### Effect of the overexpression of BAT1 and BAT2 on the flavour profile of wine

The overall fruity aroma was more prominent in the VIN13(pBAT1-s)-fermented table wine compared with the control wine but was least detected in the VIN13(pBAT2-s)-fermented table wine (Fig. 8). The peach, apricot and banana flavours were more prominent in the wines produced with the VIN13(pBAT1-s) and VIN13(pBAT2-s) transformants than in the VIN13-fermented control wine. The estery, synthetic fruit and guava characters were also more intense in the table wine fermented with VIN13(pBAT1-s). A floral aroma was only detected in the VIN13-fermented table wine.

The 'smooth' and 'herbaceous' attributes were less intense in the distillates produced with VIN13(pBAT1-s) and VIN13(pBAT2-s) than in the VIN13-fermented control wine (Fig. 9). Furthermore, the distillates produced from the base wines fermented with VIN13(pBAT1-s) and VIN13(pBAT2-s) had stronger fruity, peach, apricot, apple and synthetic fruit aromas than the control distillate.

## Discussion

In this study, we overexpressed the *Saccharomyces cerevisiae BAT1* and *BAT2* genes in the commercial wine yeast strain VIN13 and the laboratory strain BY4742. We assessed the effects of different carbon sources and amino acid concentrations, and of oxygen on the production of various aroma compounds in controlled laboratory conditions and during wine fermentations. A simplified metabolic map indicating known metabolic linkages is presented in Fig. 10. The data

**Table 9.** Statistical differences between the wines and distillates produced by the control and modified strains with respect to certain fermentation bouquet volatiles

| | Statistical difference | | | | | |
|---|---|---|---|---|---|---|
| | Colombard white wine after bottling | | Colombard base wine after alcoholic fermentation | | 70% distillate after second distillation | |
| Component | VIN13 vs. VIN13 (pBAT1-s) | VIN13 vs. VIN13 (pBAT2-s) | VIN13 vs. VIN13 (pBAT1-s) | VIN13 vs. VIN13 (pBAT2-s) | VIN13 vs. VIN13 (pBAT1-s) | VIN13 vs. VIN13 (pBAT2-s) |
| Acetic acid | 1.00 | 1.00 | 1.00 | 1.00 | 0.01 | 0.01 |
| Isobutyric acid | 1.00 | 0.02 | 0.04 | 0.01 | 1.00 | 0.01 |
| *n*-Butyric acid | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Isovaleric acid | 1.00 | 0.01 | nd | nd | nd | nd |
| *n*-Valeric acid | 0.01 | 1.00 | 1.00 | 1.00 | nd | nd |
| Propionic acid | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 2-Phenylethyl acetate | 0.01 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Diethyl succinate | 1.00 | 0.05 | 0.03 | 0.02 | 1.00 | 0.04 |
| Ethyl acetate | 0.01 | 0.07 | 1.00 | 1.00 | 1.00 | 1.00 |
| Ethyl caproate C6 | 0.05 | 0.62 | 1.00 | 1.00 | 1.00 | 1.00 |
| Ethyl caprate C10 | 0.01 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Ethyl lactate | 0.13 | 0.01 | 1.00 | 0.32 | 1.00 | 0.01 |
| Hexyl acetate | 0.04 | 0.19 | 1.00 | 1.00 | 1.00 | 1.00 |
| Isoamyl acetate | 0.05 | 0.01 | 0.01 | 0.01 | 1.00 | 1.00 |
| Methanol | 0.69 | 0.01 | 1.00 | 1.00 | 1.00 | 1.00 |
| 2-Phenylethyl alcohol | 0.01 | 0.05 | 0.27 | 0.05 | 1.00 | 1.00 |
| Propanol | 0.01 | 0.70 | 0.04 | 1.00 | 0.38 | 1.00 |
| Isobutanol | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| *n*-Butanol | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Isoamyl alcohol | 0.01 | 0.07 | 0.01 | 1.00 | 0.01 | 1.00 |
| Hexanol | 0.01 | 0.01 | 1.00 | 1.00 | 1.00 | 1.00 |

The values represented in this table are the *P*-values. If $P \leq 0.05$, the difference between the concentrations of the compound produced by the wild-type and by the modified strains is significant.
nd, not done.

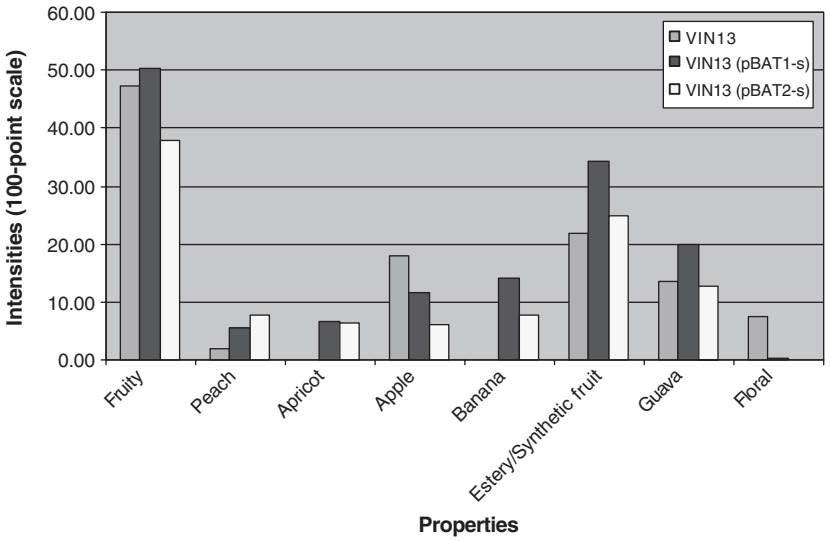


**Fig. 8.** The aroma property intensities of the Colombard wine fermented with yeast strains VIN13 (control strain) and VIN13(pBAT1-s) and VIN13(pBAT2-s) (modified strains).

show that Bat2p plays an important role in isobutanol and isobutyric acid production and, indirectly, also affects isovaleric acid production (Figs 4 and 5). Bat1p also contributes to isobutanol and isobutyric acid production, but to a lesser extent than Bat2p. These data confirm the tendencies observed by Yoshimoto *et al.* (2002) under different growth conditions and using different yeast strains. In anaerobic conditions, Bat1p can also contribute to isoamyl alcohol

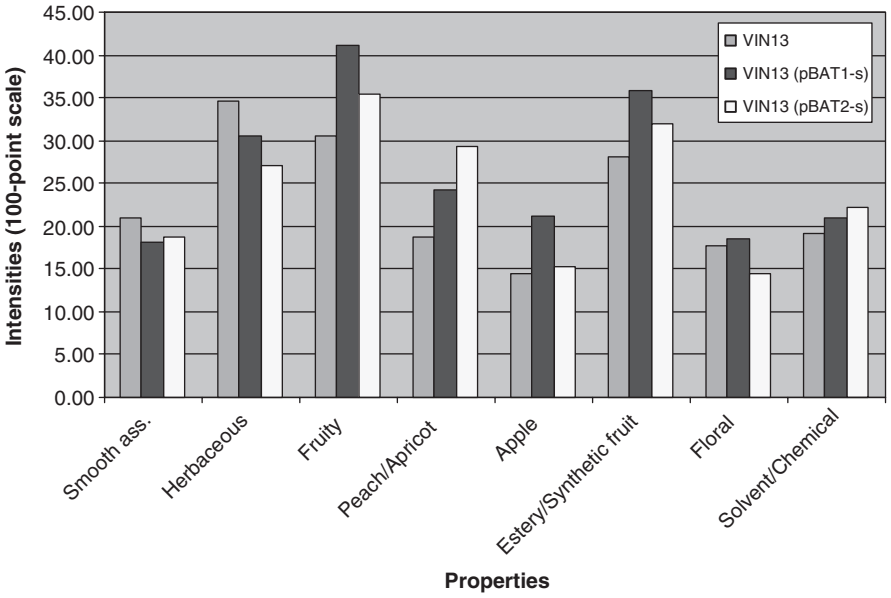


**Fig. 9.** The aroma property intensities of the distillates, distilled from Colombard base wines fermented with yeast strains VIN13 (control strain) and VIN13(pBAT1-s) and VIN13(pBAT2-s) (modified strains).

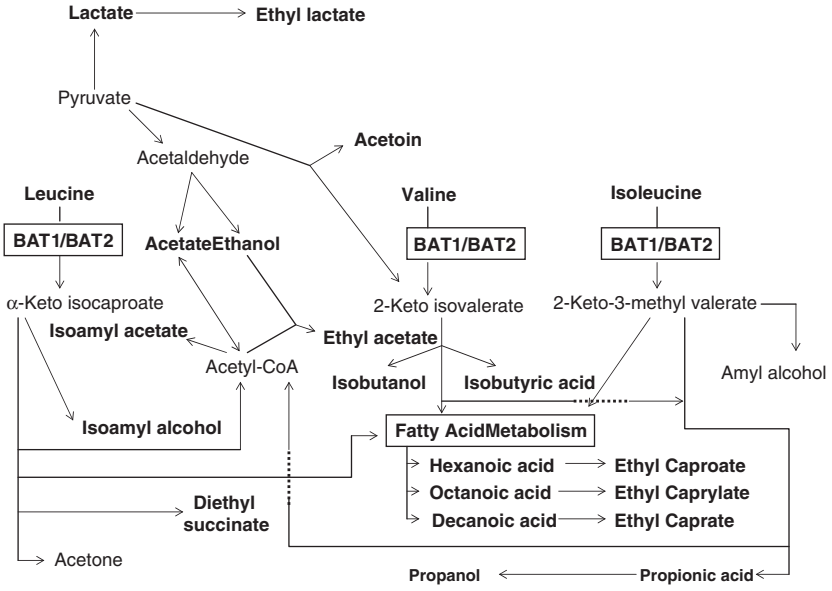


**Fig. 10.** A simplified metabolic map of yeast aroma compound production, indicating known metabolic linkages. Compounds important to this study are shown in bold.

production. Our data also show that *S. cerevisiae* uses valine as a precursor in the production of isobutanol and isobutyric acid, and that valine indirectly influences propanol and propionic acid production. The amino acids, leucine and isoleucine, directly contribute to isoamyl alcohol production and indirectly to isovaleric acid production. Leucine also indirectly contributes to 2-phenylethyl alcohol production.

To assess the effect of oxygen on higher-alcohol metabolism, we grew the yeast strains in aerobic and anaerobic conditions. Although acetate ester synthesis was greatly reduced by aeration (Fujii *et al.*, 1997), we found that the production of higher alcohols was, in some cases, affected by the presence or absence of oxygen. These differences were in some cases strain-dependent. A possible explanation for the difference between the laboratory and industrial strains might be linked to differences in their internal $NAD^+$/NADH ratios. Previously, it had been reported that quantitative differences in the redox metabolism between different *S. cerevisiae* strains could be significant (Bakker *et al.*, 2001). In addition, biomass formation is accompanied by a net production of NADH. The formation of 1 g yeast dry biomass from glucose and ammonia is accompanied by the net reduction of 10 mmol $NAD^+$ to NADH (Verduyn

*et al.*, 1990). The $OD_{600}$ was measured for the two strains during growth in the different conditions (data not shown), and the VIN13 control and transformed strains produced three times more biomass when grown aerobically than when grown anaerobically. Because the formation of higher alcohols requires NADH, this difference could explain why the VIN13 strains produced more isobutanol when grown aerobically. Indeed, the BY4742 strains produced similar amounts of biomass when grown in both conditions.

In order to assess the effect of different carbon sources on higher-alcohol production, the cells were grown in media containing glucose or raffinose. The data show that glucose does not drastically affect higher-alcohol production. But, interestingly, in the VIN13(pBAT1-s) and BY4742(pBAT1-s) strains, the fold-differences in isobutanol and isobutyric acid concentrations, compared with the VIN13 and BY4742 strains, were greater when grown in the raffinose-containing media than when grown in glucose-containing media. However, for the VIN13(pBAT2-s) and BY4742(pBAT2-s) strains, the fold-differences were greater when they were grown in the glucose media (Figs 4 and 5). The data also indicate that the VIN13(pBAT2-s) and BY4742(pBAT2-s) strains produced significantly higher isobutanol, isobutyric acid and isovaleric acid concentrations.

When the *BAT2* gene was deleted, the BY4742*bat2*Δstrain still produced the same amount of isoamyl alcohol concentrations in the SCD and SCR media, but slight decreases in concentrations were observed in the other media. Decreased amounts of isobutanol, isobutyric acid and isovaleric acid were also observed compared with the control strain BY4742. When the *BAT1* gene is overexpressed in these strains, the VIN13(pBAT1-s) and BY4742(pBAT1-s) strains produced increased amounts of isobutanol and isobutyric acid, but to a lesser extent than when *BAT2* was overexpressed in these strains. VIN13(pBAT1-s) also produced increased amounts of isoamyl alcohol concentrations under all conditions, whereas BY4742(pBAT1-s) did not show any increase in isoamyl alcohol concentrations in any of the growth conditions. With the VIN13(pBAT1-s) strain, the fold-difference in isoamyl alcohol concentrations under anaerobic conditions was greater than with the VIN13(pBAT2-s) strain under these conditions. However, aerobically the fold-differences in isoamyl alcohol concentrations with VIN13(pBAT2-s) were greater than those with VIN13(pBAT1-s). It is therefore clear that the modification of the expression levels leads to results that are dependent on the genetic background of the strains.

The assessment of the effect of amino acid concentration on the formation of higher alcohols shows that the addition of a high concentration of valine to the medium increases the isobutanol, isobutyric acid, propanol and propionic acid concentrations significantly. The addition of leucine to the medium increases isoamyl alcohol and 2-phenylethyl alcohol concentrations, and the addition of either leucine or isoleucine causes a significant increase in isovaleric acid concentration. The data therefore show that the addition of high concentrations of individual BCAAs to the growth medium results in increased levels of the corresponding higher alcohols and acids. However, increases in other higher alcohols were also observed, indicating some more complex metabolic interaction.

In order to understand better the metabolic interaction, a metabolic map (Fig. 10) was established to view the broader consequences of *BAT* gene expression levels on aroma compound production. The most significant modifications are observed for isoamyl alcohol, isovaleric acid, isobutanol and isobutyric acid concentrations. Compounds that are found further downstream in the pathway of valine and isoleucine degradation, propionic acid and propanol, appear largely unaffected by the modified expression levels of these genes.

The medium-chain fatty acids, hexanoic, octanoic and decanoic acid, show a complex pattern of production, and no clear trend can be observed between the production of such compounds and *BAT1* or *BAT2* expression. The data nevertheless clearly show that the production of these fatty acids is regulated by the availability of oxygen.

Two aroma-active compounds of which production is reduced when the *BAT* genes are overexpressed are acetoin and ethyl acetate. Acetoin is formed from one of the precursors of isobutanol and isobutyric acid. The production of acetoin is decreased when the *BAT* genes are overexpressed, with *BAT2* showing a stronger effect than *BAT1*. The increases in isobutanol and isobutyric acid concentrations might suggest an increased demand on precursor compounds, explaining the reduced formation of acetoin. Ethyl acetate, on the other hand, is formed through the reaction of acetyl-CoA with ethanol, resulting in the release of free CoA. This liberation of free CoA also accompanies the formation of isovaleric acid from isovalyryl-CoA. Ester production might therefore be inhibited to maintain an optimum ratio of free CoA to acetyl-CoA.

In the wines fermented with VIN13(pBAT1-s), a significant increase in isoamyl acetate and isoamyl alcohol concentrations was detected. These wines and distillates also showed an increase in isobutanol and isobutyric acid concentrations when compared with the control wines fermented with VIN13. However, the wines and distillates produced with VIN13(pBAT2-s) showed decreases in isoamyl acetate and isoamyl alcohol concentrations, but significant increases in isobutanol and isobutyric acid concentrations when compared with the products produced by the control strains. In the case of *BAT2* overexpression, the metabolic flux from α-keto-isovalerate is shifted in the direction of isobutanol, whereas in the *BAT1* overexpression

strain the flux is shifted towards isoamyl alcohol and subsequently isoamyl acetate production. The Colombard white wine fermented with VIN13(pBAT2-s) also showed an increase in propanol, isovaleric acid and propionic acid concentrations, respectively. The sensory evaluation showed that the wines and distillates produced with the modified strains had a stronger peach and apricot aroma. The wines also had a more prominent banana flavour, and the distillates a stronger, fruitier apple aroma than the wines and distillates produced with VIN13. These fruity aromas could originate from the corresponding aldehydes.

These sensory evaluation results were unexpected, because the wine produced with the two transformed strains contained high levels of either isoamyl alcohol or isobutanol. Although the concentrations of the respective aldehydes were not measured, it is tempting to speculate that the overexpression of *BAT1* and *BAT2* would also result in an increase in the corresponding aldehydes. The Oxford Chemicals website (www.oxfordchemicals.com) describes isobutyraldehyde, the precursor of isobutanol, as having an apple, banana and soft-fruit aroma, and isovaleraldehyde as fruity and peachy. Acetaldehyde is also described as having an apple, peach, fruity ester-like odour. These aromas, which were detected in the wines produced with the VIN13 transformants, could therefore be ascribed to the indirect increase in the corresponding aldehydes that are perceived as apple, banana, peach and soft-fruit aromas.

In conclusion, this study describes a molecular-genetic approach to investigating the effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. It is important to point out that the *BAT1*- and *BAT2*-overexpression wine yeasts described in this study are considered as genetically modified organisms (GMOs) and cannot be used commercially in the present anti-GMO climate. However, these genetically engineered prototype yeasts have been useful in demonstrating that it is indeed possible to develop seed yeasts with optimized *BAT1* and *BAT2* expression levels that would facilitate production of optimal concentrations of higher alcohols during wine fermentations. This therefore prompted us to screen natural yeasts for this characteristic. We hope to identify and develop non-GMO yeasts that can produce the desired amounts of higher alcohols, thereby assisting winemakers in their endeavour to produce wines and distillates with specific flavour profiles.

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